BIOLOGICAL PROPERTIES OF THE *X*-GENE PRODUCT OF HEPATITIS B VIRUS

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Contents

Abstract

Several biological properties of the *X*-gene product of hepatitis B virus (HBV) were investigated to begin to elucidate its function in the viral life-cycle and its contribution to the physiological consequences of HBV infection. One of these consequences is a humoral immune response mounted by the host that is directed against the *X*-gene product (HBxAg). The antigenicity of segments of HBxAg was investigated by expressing them as fusion proteins in *E. coli*. Cross-reactivity of these products was determined with antisera from an HBV infected chimpanzee and from a rabbit inoculated with HBxAg produced in *E. coli*. These polyclonal antisera contained antibodies to all segments of HBxAg, although much weaker reactivity with a fusion protein containing only the middle third of the antigen was observed with both antisera.

A striking serological feature of HBV infection is the vast amount of viral surface antigen (HBsAg) secreted from infected cells, and the contribution of HBxAg to this high level of HBsAg expression was investigated. Human hepatoma cells transfected with a plasmid construction containing the transcription units for the preS2 and surface (preS2/S) mRNAs and the X mRNA secreted HBsAg into the culture medium. A frameshift mutation in the *X* gene greatly reduced the production of HBsAg. The mutation could be complemented by cotransfection with a plasmid containing the *X* structural gene under control of the SV40 early promoter. Levels of HBsAg production were directly related to the amount of preS2/S mRNA produced showing that HBxAg can modulate expression of this gene. HBsAg polypeptides containing preS domains are secreted by HBV infected hepatocytes at a low level relative to the major-S polypeptides which contain only the surface domain. HBxAg did not influence the relative levels of the various forms of HBsAg polypeptide produced by hepatoma cells transfected with a plasmid encoding both preS domains. HBsAg production was reduced upon cotransfection of cells with a plasmid construction containing the HBV enhancer indicating that expression of HBsAg is influenced by proteins binding to this region. However, HBxAg could still exert its transactivating effect in the presence of the competitor plasmid, indicating that this effect can be mediated through DNA sequences outwith the enhancer region.

The ability of HBxAg to function as a protein serine/threonine kinase was investigated to determine whether this polypeptide may be the source of the endogenous kinase activity associated with HBV particles. In addition, this enzymatic function could account for the ability of HBxAg to modulate transcription. No protein kinase activity was detected for HBxAg expressed in *E. coli* as a fusion protein with HBcAg or for HBxAg expressed in hepatoma cells.

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Abbreviations

Abbreviations used in the text are listed below. Those used in figures are defined in the figure legend.

A:	ampere
Ad2:	adenovirus 2
Amp^r:	ampicillin resistant
AP-1:	activator protein 1
AP-2:	activator protein 2
ATP:	adenosine triphosphate
β-gal:	β-galactosidase
BCIP:	5-bromo-4-chloro-3-indoyl-phosphate
bp:	base pair
BSA:	bovine serum albumin
CAH:	chronic active hepatitis
cAMP:	cyclic adenosine monophosphate
CAT:	chloramphenicol acetyl transferase
C/EBP:	CCAAT/enhancer binding protein
CM:	carboxymethyl
CREB:	cAMP response element binding protein
CTL:	cytotoxic T-lymphocyte
DAG:	diacyl glycerol
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytosine triphosphate
DEAE:	diethylaminoethyl
DEPC:	diethyl pyrocarbonate
dGTP:	deoxyguanosine triphosphate
DHBV:	duck hepatitis B virus
DMSO:	dimethylsulphoxide
DNA:	deoxyribonucleic acid
DR:	direct repeat
DTT:	dithiothreitol
dTTP:	deoxythymidine triphosphate
E:	enhancer binding site defined by Shaul and Ben-Levy (1987)

E. coli:	*Escherischia coli*
EDTA:	diaminoethanetetra-acetic acid
EF-C:	protein that binds to polyoma virus enhancer element C
ELISA:	enzyme-linked immunosorbent assay
ER:	endoplasmic reticulum
FCS:	foetal bovine serum
FDE:	ficoll-dye-EDTA
FSBA:	p-fluorosulphonylbenzoyl 5´-adenosine
g:	acceleration due to gravity (9.8 m/s^2)
GMEM:	Glasgow modification of Eagle's medium
GnHCl:	guanidine hydrochloride
Gp-HSA:	gluteralderhyde polymerised human serum albumin
GR:	glucocorticoid receptor
GSHV:	ground squirrel hepatitis virus
GSHcAg:	ground squirrel hepatitis virus core antigen
GSHxAg:	ground squirrel hepatitis virus X antigen
HBcAg:	hepatitis B virus core antigen
HBcX:	fusion protein of HBcAg and HBxAg
HBeAg:	hepatitis B virus e antigen
HBS:	hepes-buffered saline
HBsAg:	hepatitis B virus surface antigen
HBV:	hepatitis B virus
HBxAg:	hepatitis B virus X antigen
HCC:	hepatocellular carcinoma
HEPES:	N-2-hydroxyethylpiperazine-*N´*-2-ethanesulphonic acid
HGH:	human growth hormone
HHV:	heron hepatitis virus
HIV:	human immunodeficiency virus
HNF-1:	hepatocyte nuclear factor 1
HPRT:	hypoxanthine ribosyl transferase
HSA:	human serum albumin
HSV:	herpes simplex virus
HTLV:	human T-lymphotropic virus
IgG:	immunoglobulin class G

IL-2:	interleukin 2
IPTG:	isopropyl-B-D-thiogalactopyranoside
kb:	kilobase pairs or kilobases
kD:	kilodaltons
L-broth:	Luria broth
LTB:	low-Tris buffer
LTR:	long terminal repeat
MHC:	major histocompatibility complex
MMTV:	mouse mammary tumour virus
MOPS:	3-(*N*-morpholino) propane-sulphonic acid
mRNA:	messenger ribonucleic acid
MSV:	murine sarcoma virus
NBT:	nitro-blue-tetrazolium
NF-1:	nuclear factor 1
NP40:	nonidet P40
Oct-1:	octamer-binding factor 1
OD:	optical density
OLB:	oligo-labelling buffer
ORF:	open reading frame
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate-buffered saline
PCI:	phenol/chloroform/iso-amyl alcohol
PCR:	polymerase chain reaction
PEG:	polyethylene glycol
pk:	protein kinase
PKA:	protein kinase A
PKC:	protien kinase C
PMA:	phorbol 12-myristate 13-acetate
PMSF:	phenylmethylsulphonyl fluoride
preC:	pre-core
preS:	pre-surface
Rb:	retinoblastoma-gene product
RF:	replicative form (of bacteriophage M13 DNA)
RNA:	ribonucleic acid

RSV:	Rous sarcoma virus
SDM:	site directed mutagenesis
SDS:	sodium dodecyl sulfate
Sp1:	sephacryl/phosphocellulose fraction protein 1
SSC:	solution containing NaCl and tri-sodium citrate
STE:	buffer containing Tris-HCl, EDTA and NaCl
SV40:	simian virus 40
Tag:	SV40 large T-antigen
TAR:	transacting response element
taxRE:	HTLV1 $p40^{tax}$ response element
TBE:	buffer containing Tris-HCl, boric acid and EDTA
TE:	buffer containing Tris-HCl and EDTA
TEMED:	*N,N,N,N´*-tetramethyl-ethylenediamine
Tet^r:	tetracycline resistant
TFIID:	RNA polymerase II transcription factor D
TFIIIA:	RNA polymerase III transcription factor A
TFIIIC:	RNA polymerase III transcription factor C
TGT3:	TGTTT sequence in hepatitis B virus enhancer region
tk:	herpes simplex virus thymidine kinase
TM:	buffer containing Tris-HCl and $MgCl_2$
T_m:	melting temperature of DNA duplex
Tris-HCl:	Tris (hydroxymethyl) aminoethane hydrochloride
Triton X100:	octylphenoxy polyethoxyethanol
TS:	buffer containing Tris-HCl and NaCl
TS/milk:	solution of powdered milk in TS.
Tween:	polyoxyethylene-sorbitan monolaurate
UE1:	upstream enhancer binding region 1
UE2:	upstream enhancer binding region 2
UE3:	upstream enhancer binding region 3
U:	unit of enzymatic activity
V:	volt
WHV:	woodchuck hepatitis virus
WHxAg:	woodchuck hepatitis virus X antigen
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino acid symbols:

Amino acid	Three-letter symbol	Single-letter symbol
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamine	Gln	Q
glutamic acid	Glu	E
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

CHAPTER 1: Introduction

1.1 Hepatitis B Disease Characteristics

Hepatitis B is an infectious liver disease of man. The serological source of infection was affirmed by the discovery of a novel antigen in patient sera (Blumberg *et al.*, 1965; Prince, 1968) eventually shown to be a viral gene-product. The causative agent, the hepatitis B virus (HBV), was discovered by Dane *et al.* (1970) who observed the virion, subsequently referred to as the Dane particle, in electron micrographs of serum from patients with hepatitis B. The virus can be transmitted vertically from mother to newborn baby or horizontally through sexual contact or blood contact. The disease is also referred to as serum hepatitis due to the blood to blood aspect of its transmission.

HBV exhibits narrow host range, infecting only humans and higher primates (Maynard *et al.*, 1972, Barker *et al.*, 1973). In the infected host, the virus shows strong tissue tropism primarily infecting liver cells (Berquist *et al.*, 1975). HBV DNA has been detected in low copy number in cells other than hepatocytes, most commonly blood leukocytes and bone marrow, indicating limited non-hepatic infection (Elfassi *et al.*, 1984; Laure *et al.*, 1985; Korba *et al.*, 1986). However, the biological consequences of this observation are unclear.

The disease state induced by HBV infection is manifested in several ways characterised by the extent of liver inflammation and damage and viral persistence. Variations in the pathogenic effects of HBV infection are attributed to differences in the host's immune system (reviewed by Dienstag, 1984). The majority of individuals infected with HBV experience acute infection associated with varying amounts of hepatocyte necrosis which is manifest by increased serum transaminase activity and patient jaundice. The host mounts an immune attack on the infection, producing antibodies to the viral antigens (figure 1.1). Evidence that HBV is not directly cytopathic (reviewed by Ferrari *et al.*, 1988) supports the hypothesis that hepatocyte necrosis results from the effects of the cytotoxic T-lymphocytes (CTLs) specific for proteolytic fragments of HBV antigens expressed on the surface of infected cells in association with major histocompatibility complex (MHC) class I antigens (Mondelli *et al.*, 1982; reviewed by Dienstag, 1984).

While in the majority of cases, acute infection is resolved by complete clearance of the virus and the development of immune memory to counter reinfection, a small percentage of hosts develop a fulminant disease state resulting in severe liver dysfunction and leading to high mortality (O'Grady *et al.*, 1986). 5-10% of infected adults develop chronic HBV infection (Hoofnagle and Alter, 1984) characterised by the persistence of viral antigens

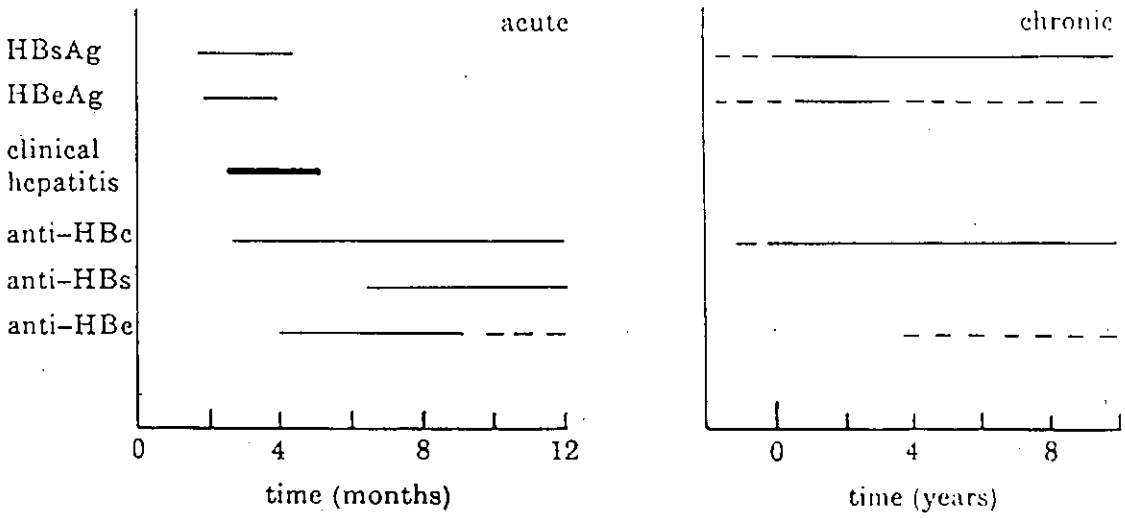


FIGURE 1.1 Serological profile of HBV infection.

Temporal appearance of HBV antigens and antibodies to them during acute and chronic infection. Variations in the time at which the various markers appear in individual cases are indicated by dashed lines. Adapted from Murray, 1987.

in patient serum (figure 1.1). This disease state may continue after integration of HBV DNA into the host-cell chromatin where transcription of viral antigens may continue in the absence of virion production. Chronic infection can give rise to substantial liver cirrhosis but may also lead to the development of a carrier state in which viral antigens are present in the serum in the absence of other pathological features of the disease. Chronically infected patients are predisposed to developing hepatocellular carcinoma (HCC) (Szmuness,1978) with more than 100-fold greater chance of developing this neoplasia than non-infected individuals (Beasley, 1981). Hepatitis B is a worldwide health problem because of the number of chronically infected people in the world estimated at 2×10^8 (Szmuness,1978).

1.2 Virion and Genome Structure

HBV is the prototype member of the hepadnavirus family which includes hepatitis viruses isolated from the woodchuck (WHV) (Summers *et al.*, 1978), ground squirrel (GSHV) (Marion *et al.*, 1980), Pekin duck (DHBV) (Mason *et al.*, 1980) and grey heron (HHV) (Sprengel *et al.*, 1988). This taxonomy is derived from the relative hepatropism of virus family members, their common virion morphology, common genome size, structure and organization, and common mechanism of genome replication which proceeds through reverse transcription of an RNA intermediate in a manner analogous to retroviruses.

In 1970, Dane *et al.* observed the 42 nm hepatitis B virion in electron micrographs of serum from infected patients. The virion has an outer envelope of 7 nm thickness which is lipid bilayer derived from the endoplasmic reticulum (ER) membrane of the host hepatocyte (Patzer *et al.*, 1986). Embedded in this envelope are molecules of surface antigen (HBsAg) encoded by the viral genome (figure 1.2a). Within the envelope of the virus particle is the nucleocapsid. This 27 nm protein core houses the viral genome and is composed of 180 subunits of the virally encoded core antigen (HBcAg) arranged in icosahedral conformation with T=3 symmetry (Onodera *et al.*, 1982). A protein-kinase activity is associated with the nucleocapsid with the capacity to phosphorylate HBcAg (Albin and Robinson, 1980, Feitelson *et al.*, 1982; Gerlich *et al.*, 1982; see Chapter 5 for further discussion). The viral genome was first detected as the endogenous template of a DNA polymerase activity displayed by immunoprecipitated virion core particles (Robinson and Greenman, 1974).

A large body of work led to the elucidation of the structure of the HBV genome

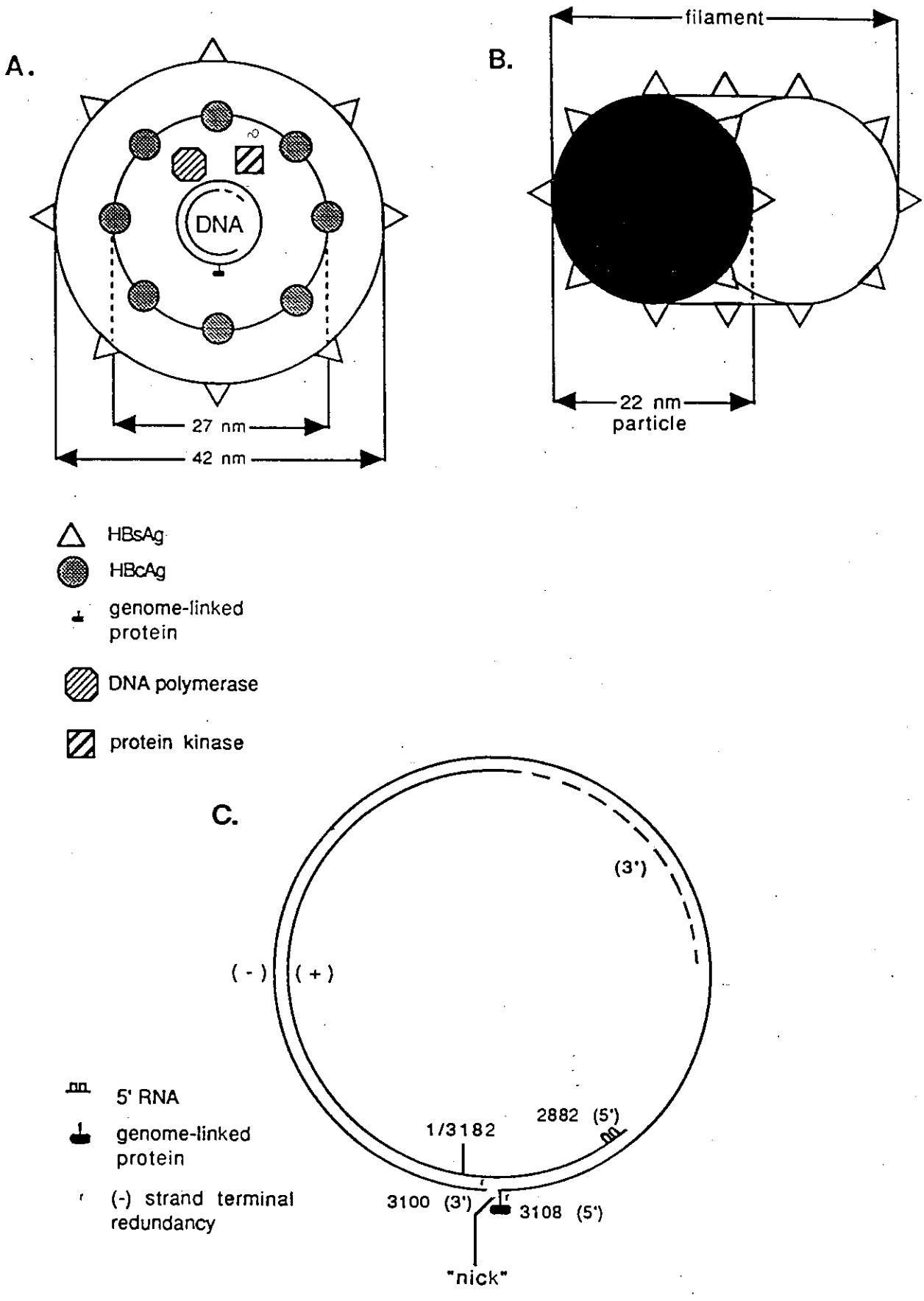
FIGURE 1.2 Structure of the HBV virion, HBsAg particles and the viral genome.

A) The HBV virion (Dane particle). The viral genome and virion associated enzymatic activities are located within the 27 nm nucleocapsid composed of HBcAg. The nucleocapsid is enveloped in host derived lipid into which HBsAg is embedded forming a virus particle with a diameter of 42 nm.

B) Secreted forms of HBsAg. Filaments and spherical particles that are composed of HBsAg and host-derived lipid are secreted from HBV infected cells. Both forms have a diameter of 22 nm.

C) The HBV genome. The viral genome is a partially double-stranded, circular molecule with a protein covalently attached to the 5´ end of the minus strand and an oligoribonucleotide linked to the 5´ end of the plus strand.

Adapted from Jackson (1987).


A.
DNA
27 nm
42 nm
B.
filament
22 nm
particle
HBsAg
HBcAg
genome-linked protein
DNA polymerase
protein kinase
C.
(3')
(-)
(+)
1/3182
2882 (5')
3100 (3')
3108 (5')
"nick"
5' RNA
genome-linked protein
(-) strand terminal redundancy

(reviewed by Robinson, 1977; figure 1.2c). The circular, partially double stranded, DNA genome contains two strands of unequal length. The long strand serves as template for viral mRNA transcription and, by convention, is termed the minus strand. This strand is 3190 bases long in the HBV isolate used in these studies (Burrell *et al.*, 1979; Pasek *et al.*, 1979; Pugh *et al.*, 1986). The numbering system employed in this thesis fixes nucleotide number 1 at the start of the coding sequence for HBcAg (Pasek *et al.*, 1979; see Appendix I). The minus strand of HBV DNA is not a covalently closed circle as it contains a nick yielding 5′ and 3′ termini which have been mapped to nucleotide positions 3100 and 3108 respectively (Will *et al.*, 1987). The eight nucleotide terminal redundancy forms a single-stranded tail at the 5′ end of the strand to which a protein is covalently attached (Gerlich and Robinson, 1980). The minus strand contains a 10 bp sequence repeated at two places (direct repeat (DR) 1: 3106-3116 and DR2: 2872-2882). These repeats and the protein attached to the 5′ end are significant features in replication of the viral genome (see section 1.5.2).

The plus strand of DNA in the HBV virion is incomplete with a variable 3′ end leaving a single-stranded gap in the genome. The size of the gap averages one kilobase (kb), with all HBV DNA molecules in a given population being double stranded 5′ to nucleotide position 1420 on the plus strand (Delius *et al.*, 1983). The endogenous DNA polymerase uses the 3′ hydroxyl-group of the plus strand as a primer to close the single stranded gap and make fully double stranded DNA molecules. The 5′ end of the plus strand has been mapped to position 2882 (Will *et al.*, 1987), and the HBV genome is held in circular conformation because the 5′ end of the plus strand does not correspond with the 3′ end of the minus strand but overlaps and pairs with approximately 220 bases at the 5′ end of the minus strand (Sattler and Robinson, 1979). Covalently attached to the 5′ end of the plus strand is a capped oligoribonucleotide of about 17 bases in length (Will *et al.*, 1987) which primes synthesis of the plus strand during viral replication (see section 1.5.2).

1.3 Genome Organisation and Viral Polypeptides

The genome of several HBV isolates has been cloned in *E. coli* and the nucleotide sequence determined (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1980; Fujiyama *et al.*, 1983; Ono *et al.*, 1983; Kobayashi and Koike, 1984; Okamoto *et al.*, 1987). Productive infection was established in chimpanzees by inoculation with cloned HBV

DNA intrahepatically or by intrahepatic injection of autologous hepatocytes transfected with cloned HBV DNA (Will *et al.*, 1985). This demonstrated that no essential viral sequences had been altered during cloning and that the viral genome structural features were not necessary for infectivity. The HBV sequence revealed 4 major open reading frames (ORFs) in the 3.2 kb minus strand using all three translational frames (figure 1.3). The ORFs include preS/surface, preC/core, polymerase and X. These four genes are conserved in all of the mammalian hepadnaviruses, while the avian viral genomes lack an ORF with homology to the *X*-gene (Mandart *et al.*, 1984; Sprengel *et al.*, 1988). The hepadnaviruses provide a superlative example of the genetic economy of viruses, as each nucleotide in the genome is part of at least one ORF.

In the past, the molecular analysis of HBV has been limited to the animal models and to the expression of viral antigens encoded by cloned HBV DNA in a variety of systems. Recently, several laboratories have demonstrated that certain hepatoma cell lines are competent for the expression of all of the HBV genes from their native transcriptional control elements and for the production of virus particles (Sureau *et al.*, 1986; Chang *et al.*, 1987; Sells *et al.*, 1987; Tsurimoto *et al.*, 1987; Yaginuma *et al.*, 1987b; Shih *et al.*, 1989).

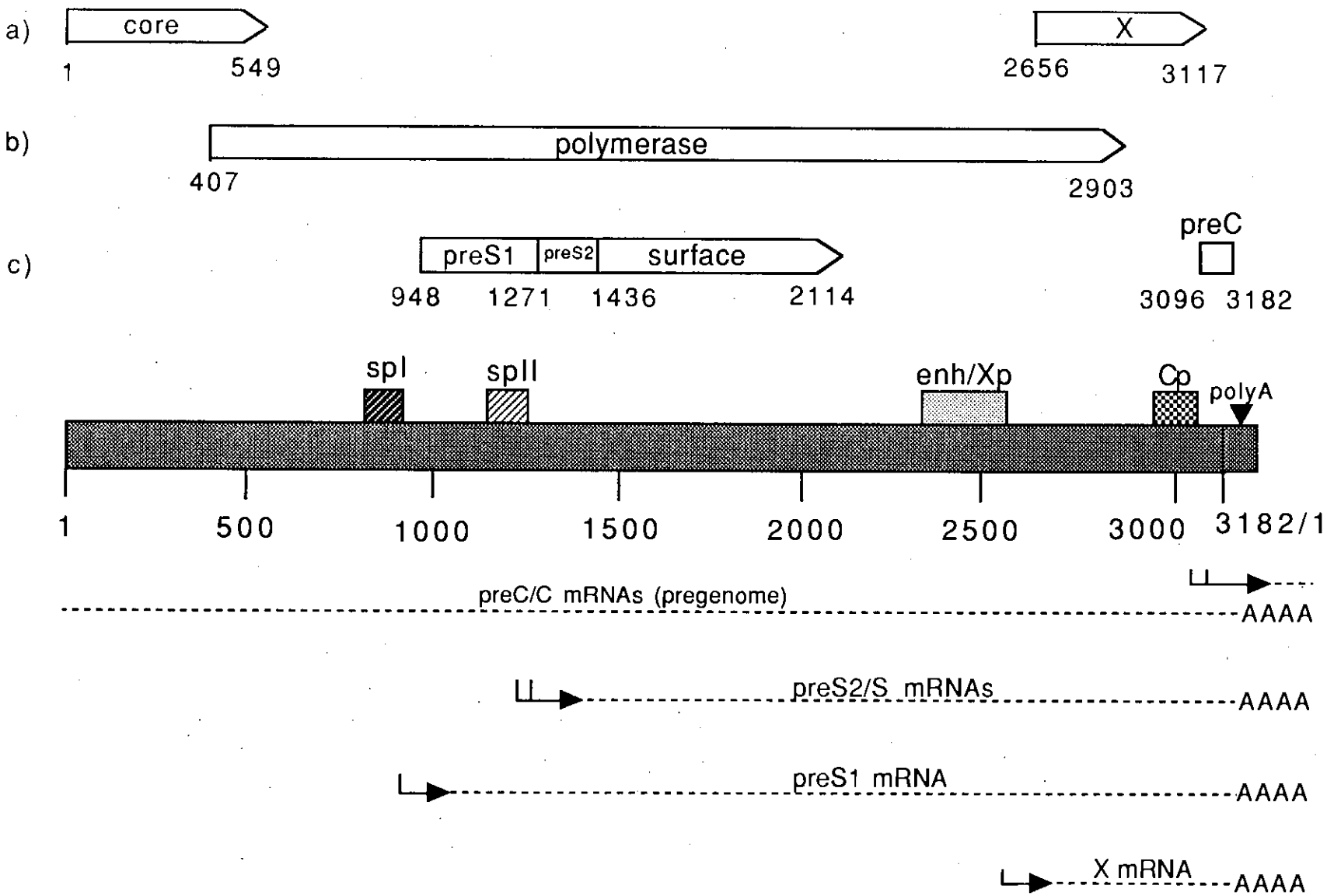
1.3.1 HBsAg

In addition to 42 nm virus particles, electron micrographs of patient serum reveal an abundance of 22 nm spherical particles and rod shaped filaments with the same 22 nm diameter and variable length (figure 1.2b). These particles, which are present in vast excess over the 42 nm virions (Dane *et al.*, 1970), are composed exclusively of HBsAg and host derived lipid, and they may serve as "decoy virions" to bind virus neutralising, anti-HBsAg antibodies produced during infection (Murray, 1987). The characterisation of anti-HBsAg antibodies has defined distinct epitopes on HBsAg including a major antigenic determinant (*a*) common to all HBV isolates and two sets of mutually exclusive subtype determinants (*d/y*) and (*r/w*) giving rise to four major antigenic subtypes of HBV: *adr*, *adw*, *ayr* and *ayw* (LeBouvier, 1971; and Bancroft,1972). The cloned HBV DNA used in these studies was isolated from a virus subtype designated *adyw*. The donor serum from which virus was isolated displayed this compound virus subtype (Burrell *et al.*, 1979), which may be due to infection by more than one virus subtype.

The common and subtype-specific antigenic determinants are located within the

FIGURE 1.3 HBV genome organisation and viral transcripts.

A linear representation of the HBV genome is shown. Nucleotide positions of ORFs in three translational reading frames (a,b,c) are shown. Numbers indicate the first and last coding nucleotides. Transcriptional control regions are shown as boxes and are abbreviated as follows: spI = preS1 promoter; spII = preS2/S promoter; enh/Xp = enhancer/X promoter; Cp = core promoter. ▼ Indicates HBV polyadenylation signal at nucleotide position 16. Viral transcripts are represented by dashed lines with arrows highlighting transcription initiation sites.


a)
core
1
549
X
2656
3117
b)
polymerase
407
2903
c)
preS1
preS2
surface
preC
948
1271
1436
2114
3096
3182
spI
spII
enh/Xp
Cp
polyA
1
500
1000
1500
2000
2500
3000
3182/1
preC/C mRNAs (pregenome)
AAAA
preS2/S mRNAs
AAAA
preS1 mRNA
AAAA
X mRNA
AAAA

principle forms of HBsAg (major-S), which are a protein of 24 kilodaltons (p24) and its glycosylated derivative, gp27 (Peterson *et al.*, 1977; Peterson, 1981), referred to henceforth as the major-S polypeptides. These two proteins are present in nearly equal quantities in the virion envelope and in subviral HBsAg forms (Heerman *et al.*, 1984). Partial amino- and carboxy-terminal sequence of the major-S polypeptides (Peterson, 1977) revealed that they are encoded by nucleotide positions 1437-2114 (the surface ORF) yielding polypeptides of 226 amino acids in length with gp27 glycosylated at asparagine residue 146 (figure 1.4). Larger polypeptides are present in all morphological forms of HBsAg. All of the polypeptides cross-reacted with antiserum raised against p24 indicating that they are coterminal at the carboxyl-ends with the major-S proteins (Heerman *et al.*, 1984). The middle-S polypeptides (gp33 and gp36) contain an additional 55 residues at their amino-terminus, which are encoded by the preS2 ORF. Both forms of middle-S polypeptide are glycosylated at amino acid residue number 4 of the preS2 domain, while the size difference between the two forms is due to the differential glycosylation at the site within the surface domain (Stibbe and Gerlich, 1983). The large-S polypeptides (p39 and gp42) are encoded by the preS1, preS2 and surface ORFs, fusing 163 residues to the amino-terminus of the major-S proteins. The coding capacity of the preS1 ORF varies among HBV subtypes with the *adw*, *adr*, and *ayr* subtypes containing an additional 11 codons at the 5′ end. The size difference between the large-S polypeptides is again due to differential glycosylation at the site within the surface domain. As the two forms of major-, middle-, or large-S arise from differential glycosylation of the same site it is likely that whatever dictates the relative amounts of p24 and gp27 also carries over to middle- and large-S proteins.

In addition to potential modification by glycosylation, both forms of large-S polypeptide are acylated, with myristic acid covalently bound to the amino-terminal glycine residue (Persing *et al.*, 1987). All hepadnaviral large-S proteins contain the sequence methionine-glycine at the amino-terminus, and it is proposed that the amide linkage to myristate is formed with the exposed amino-group of the glycine residue following cleavage of the initiator methionine.

Monoclonal antibodies that react exclusively with either middle- or large-S polypeptides in immunoblot analysis, have been produced by inoculation of mice with HBV virion (Heerman *et al.*, 1984; Heerman *et al.*, 1987). This demonstrated that the minor forms of HBsAg are present in the virion and that preS1 and preS2 domains are exposed on the surface of the envelope where they are capable of eliciting an antibody

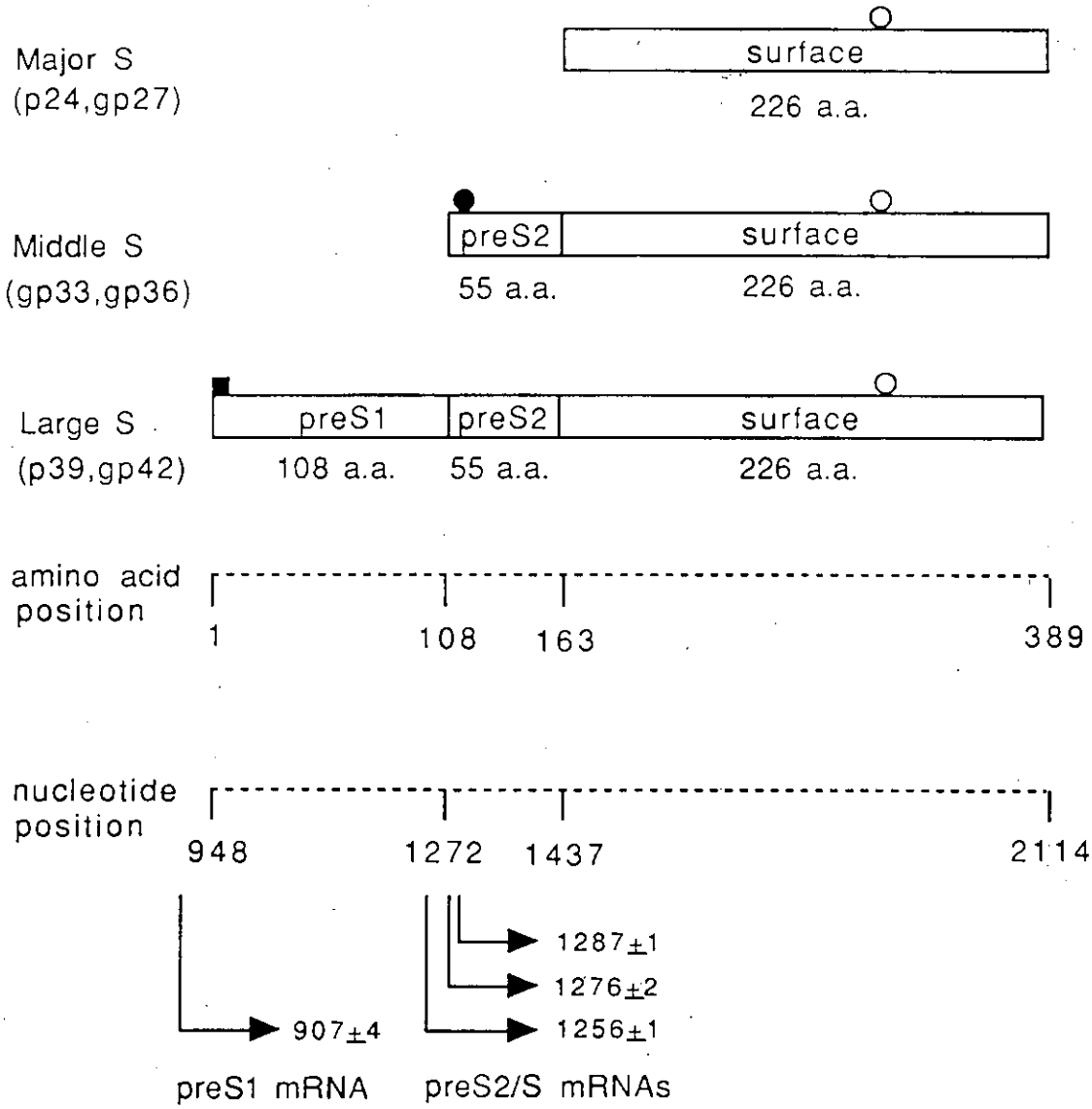


FIGURE 1.4 HBsAg forms.

Surface antigen domains contained in major-, middle-, and large-S polypeptides are indicated. The filled circle represents the glycosylated amino acid residue in the preS2 domain, while an open circle represents the residue in the surface domain that is variably glycosylated. The filled square indicates the myristoylated residue in the preS1 domain. Transcriptional start sites are represented by arrows, and the numbers indicate the nucleotide positions mapped by Yaginuma *et al.* (1987b). Adapted from Neurath (1989).

response. Using antiserum raised against p24, the polypeptide composition of all three morphological forms of HBsAg was determined by immunoblotting (Heerman *et al.*, 1984). The large-S polypeptides comprise 1-2% of the total envelope proteins in 22 nm spherical particles, while HBsAg filaments and the virion envelope contain up to 20% of these species. The middle-S polypeptides comprise 5-10% of total HBsAg polypeptides in all three forms. The relative amounts of HBsAg polypeptides are controlled at the transcriptional level and by the differential use of start codons within a particular transcript (see section 1.4.1).

HBsAg has been expressed in high yield in yeast (reviewed by Murray, 1987) and in a variety of mammalian cell lines under control of native or heterologous transcriptional control elements (reviewed by Acs and Price, 1990). Studies in both systems indicated that the major-S polypeptide alone contains all of the information necessary for correct assembly and secretion of 22 nm spherical particles (Valenzuela *et al.*, 1982; Persing *et al.*, 1985). Examination of HBV infected hepatocytes (Gerber *et al.*, 1974) or of stably transfected cells producing HBsAg (Patzer *et al.*, 1986) by electron microscopy revealed that 22 nm particles are assembled in the ER and secreted via the constitutive secretory pathway through which virion is also secreted (Yamada *et al.*, 1982; see section 1.5.3). Particle assembly initiates with the translocation of HBsAg monomers into the ER membrane (Eble *et al.*, 1986). Transmembrane molecules aggregate in the plane of the membrane and bud off into the lumen of the ER. Secretion of HBsAg particles appears to be less efficient than that of host-cell proteins with the rate limiting step being the transfer from the ER to the Golgi apparatus (Patzer *et al.*, 1984).

Transfer of HBsAg from the ER through the rest of the secretory pathway is influenced by the presence of large-S forms. Large-S polypeptides, overexpressed in mammalian cells under control of a heterologous promoter, are not secreted but are retained in the ER. In addition, overexpression of large-S proteins can inhibit secretion of middle- and major-S polypeptides when the different forms are expressed from the same plasmid construction or from cotransfected plasmids (Laub *et al.*, 1983; Persing *et al.*, 1986; McLachlan *et al.*, 1987; Ou and Rutter, 1987; Molnar-Kimber *et al.*, 1988). The extent of inhibition in stably transfected cells correlated with the proportion of total HBsAg constituted by large-S polypeptides (Ou and Rutter, 1987). This correlation was also observed in *Xenopus* oocytes injected with a constant amount of mRNA encoding middle- and major-S polypeptides and varying amounts of mRNA encoding large-S forms (Standring *et al.*, 1986). Large-S polypeptides are expressed at a relatively low level

during infection compared to the heterologous systems representing only 1-5% of the total pool of HBsAg (Heerman *et al.*, 1984). Inhibition of HBsAg secretion was not observed upon expression of preS1 sequences under control of the native promoter in COS (monkey kidney) cells (Siddiqui *et al.*, 1986), and there is an abundance of HBsAg secreted during natural infection. However, the preferential location of large-S polypeptides in the virion envelope and HBsAg filaments suggests that their retention in the ER may contribute to the formation of more complicated structures.

The sequences required for ER retention have been investigated by deletion analysis of the large-S polypeptides (Kuroki *et al.*, 1989; Yu, 1991). A mutant in which only the first 19 amino acid residues of the preS1 domain were fused to the preS2 and surface domains was retained in the ER, whereas further deletion of amino acids 9-19 resulted in efficient secretion of the product (Kuroki *et al.*, 1989). This indicated that myristoylation at amino acid residue 2 (glycine) was not involved in ER retention, which was confirmed by mutating this particular residue to alanine in the full length large-S polypeptide thereby abolishing acylation but not affecting ER retention. The presence of an ER retention signal within the first 19 amino acid residues of the preS1 domain was confirmed by Yu (1991), who noted, however, that an additional deletion of residues 66-117 of the preS1 domain restored wild-type characteristics to the mutant protein. The mechanism of ER retention of both the wild type and double-mutant large-S polypeptides remains to be elucidated.

Vaccine development against HBV has focused on priming the immune system to produce antibodies against HBsAg. The serum derived vaccine consists of purified 22 nm particles (Hilleman *et al.*, 1978), and has proven safe and effective (Szmuness *et al.*, 1980). Production of the serum derived vaccine is costly due to the necessary purification and testing procedures, and its use carries the perceived risk of contamination with the human immunodeficiency virus (HIV). Genetically engineered vaccines utilise HBsAg particles consisting of major-S polypeptide (p24) produced in yeast (*S. cerevisiae*). The recombinant antigen was shown to protect chimpanzees upon subsequent challenge with HBV (Murray *et al.*, 1984), and is now in general use for vaccination of human populations.

Studies with the various surface antigen species have revealed the importance of the preS regions in the immune response to HBV infection. Antibodies against both the preS1 and preS2 regions are produced during acute infection (Neurath *et al.*, 1985; Klinkert *et al.*, 1986). While the antigenic determinants (or epitopes) within the major-S polypeptide are dependent on conformation, the preS regions contain continuous epitopes

whose binding to antibody is independent of disulphide linkages (Neurath *et al.*, 1985). Immunisation of mice with genetically engineered 22 nm particles composed of middle- and major-S polypeptides has shown that the preS2 region is significantly more immunogenic than the S region (Milich *et al.*, 1985). In this system, the antibody responses to the preS2 epitopes regularly exceeded the anti-S response. In the same study, the immune response to HBsAg particles containing middle- and major-S polypeptides was investigated in mice from a strain that does not produce a significant antibody response when inoculated with particles consisting of only major-S protein. Immunisation with the mixed particles elicited antibody production to both preS and S epitopes. This phenomenon can be explained by efficient stimulation of T-helper cells by the preS2 region. (See below, section 1.3.2, for a more detailed discussion of T-help specific for one component of a polypeptide or particle stimulating antibody production against another.) In support of this hypothesis, T-helper cell recognition sites have been identified within the preS2 domain (Milich *et al.*, 1986b, 1990). T-helper cell recognition sites have also been identified in the preS1 region, and synthetic peptides representing the amino acid sequence at these sites can stimulate T-helper cells for antibody production to epitopes within the preS1, preS2 and S regions of HBsAg (Milich *et al.*, 1986a,1987a).

The preS domains of HBsAg are important for viral uptake into hepatocytes (see section 1.5.1), and synthetic peptides representing amino acid sequence from the preS2 region can elicit an HBV-neutralising antibody response in chimpanzees (Itoh *et al.*, 1986; Neurath *et al.*, 1986a). The growing evidence for the importance of the preS regions in viral function and host immune response argues for their inclusion in HBV vaccines.

1.3.2 HBcAg

The sequence of the core ORF spans nucleotide positions 1 to 549 capable of encoding a protein of 183 amino acids and approximately 21 kilodaltons (kD). HBcAg itself is not detectable in the serum of infected individuals, although, during recovery from acute infection, an antibody response is mounted against this antigen. The reactivity of antibodies raised against HBcAg is conformation dependent, as denaturation of core changes its antigenic specificity to HBeAg (Ohori *et al.*, 1980). HBeAg is found in large quantities in the serum of HBV infected individuals and the appearance of antibodies specific for this antigenic form of HBcAg indicates low infectivity of serum and is prognostic of viral clearance (Overby *et al.*, 1983). Evidence that HBeAg may be a

proteolytic cleavage product of HBcAg was supplied by MacKay *et al.* (1981), who converted HBcAg synthesized in *E. coli* to HBeAg by treatment with pronase and β-mercaptoethanol. Amino acid sequence analysis of serum derived HBeAg showed that it is coterminal with HBcAg at the amino-terminus while 34 amino acid residues from the carboxyl-terminus of HBcAg were missing in HBeAg (Takahashi *et al.*, 1983).

HBcAg encoded by cloned HBV DNA has been produced in a variety of expression systems, initially in *E. coli* (Pasek *et al.*, 1979; Stahl *et al.*, 1982) and subsequently in mammalian cells (Gough and Murray, 1982; Will *et al.*, 1984; Roossinck *et al.*, 1986; McLachlan *et al.*, 1987; Ou *et al.*, 1986; Roossinck and Siddiqui, 1987; Jean-Jean *et al.*, 1989a), yeast (Kniskern *et al.*, 1986; Miyanohara *et al.*, 1986), *Xenopus* oocytes (Standring *et al.*, 1988), insect cells (Lanford and Notvall, 1990) and *in vitro* (Weimer *et al.*, 1987; Garcia *et al.*, 1988). The core ORF in the HBV genome is proceeded by an initiation codon in the same translational reading frame, 86 nucleotides upstream of the HBcAg start site. This region, from nucleotide position 3096-3182, is termed pre-core (preC) and encodes a highly hydrophobic domain. Expression of the core gene with or without the preC region in a variety of eukaryotic systems has revealed that secreted HBeAg is produced from a polypeptide precursor initiating at the preC start codon (McLachlan *et al.*, 1987; Ou *et al.*, 1986; Weimer *et al.*, 1987; Standring *et al.*, 1988). The 29 residue preC signal sequence directs translocation of the preC/core polypeptide across the ER membrane and is cleaved after residue 19 (Garcia *et al.*, 1988). Proteolytic processing of the carboxyl-terminus results in secretion of the mature 17 kD HBeAg polypeptide. The preC region is required for cell-surface expression of a preC/core gene-product in cultured human hepatoma cells (HepG2) infected with a vaccinia virus recombinant carrying this gene (Schlicht and Schaller, 1989).

Comparison of the predicted amino acid sequence of HBcAg to a protein sequence database revealed a sequence homologous to the active site of aspartyl-proteases which could potentially carry out self-cleavage during biosynthesis of HBeAg (Miller, 1987). However, in HepG2 cells transiently transfected with the complete HBV genome, mutation of the essential aspartate residue within the protease-like sequence did not affect secretion of HBeAg indicating that this function can be carried out by a cellular enzyme (Nassal *et al.*, 1989).

Examination of the predicted amino-acid sequence of HBcAg revealed a highly-charged, protamine-like sequence at the carboxyl-terminus consisting of four clusters of arginine residues. Petit and Pillot (1985) demonstrated that HBcAg isolated from infected

hepatocytes and transferred to nitrocellulose membrane after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), bound non-specific DNA but showed stronger affinity for HBV DNA. There are conflicting reports, however, about the importance of the protamine-like region in mediating nucleic acid binding. Both recombinant HBcAg produced in *E. coli* and serum derived HBeAg, immobilised on nitrocellulose membrane, bound HBV DNA (Matsuda *et al.*, 1988), while recombinant HBeAg did not (Gallina *et al.*, 1989). The two HBeAg preparations differed in the 10 amino acid residues of the preC region and amino acids 143-149 that are present in serum derived HBeAg but were absent in the recombinant antigen, and it is possible that those differences account for the discrepancy in results.

The highly basic carboxyl-terminus of HBcAg also resembles the signals that direct nuclear localisation for several viral and cellular proteins, for example, adenovirus E1a (Lyons *et al.*, 1987), SV40 T-antigen (Kalderon *et al.*, 1984) and glucocorticoid receptor (Picard and Yamamoto, 1987). Viral nucleocapsids can be detected in the nucleus of HBV infected hepatocytes (Ray *et al.*, 1976; Chu and Liaw, 1987). However, expression of the core gene in cultured mammalian cells has yielded disparate results, with observations of nuclear localisation (McLachlan *et al.*, 1987; Eckhardt *et al.*, 1991), cytoplasmic localisation (Jean-Jean *et al.*, 1989a; Ou *et al.*, 1989; Yeh *et al.*, 1990), or both (Roossinck *et al.*, 1987). In expression systems where HBcAg itself was cytoplasmic, nuclear localisation has been observed for a protein representing the precursor of HBeAg in which the 19 amino acid residues of the signal sequence have been cleaved off, but the protamine-like domain remains (Ou *et al.*, 1989; Yeh *et al.*, 1990). These forms have been observed from *in vitro* translation of preC/core mRNA in the presence of pancreas microsomal vesicles (Garcia *et al.*, 1988), and presumably represent aborted membrane translocation events. Eckhardt *et al.* (1991) observed nuclear localisation of HBcAg itself and demonstrated the importance of the arginine clusters in mediating nuclear translocation; the importance of this region was also shown for nuclear localisation of the HBeAg precursor (Yeh *et al.*, 1990). However, Yeh and his colleagues argue that the 10 amino terminal-residues remaining after cleavage of the preC signal peptide are also required, as HBcAg itself is not localised to the nucleus in their expression system.

Several lines of evidence have led to the investigation of HBcAg as a candidate component of HBV vaccines. The humoral response to the viral surface antigen can vary considerably from patient to patient during infection or after vaccination ranging from high- to non-responder phenotypes (Hoofnagle *et al.*, 1978). In contrast, high titres of

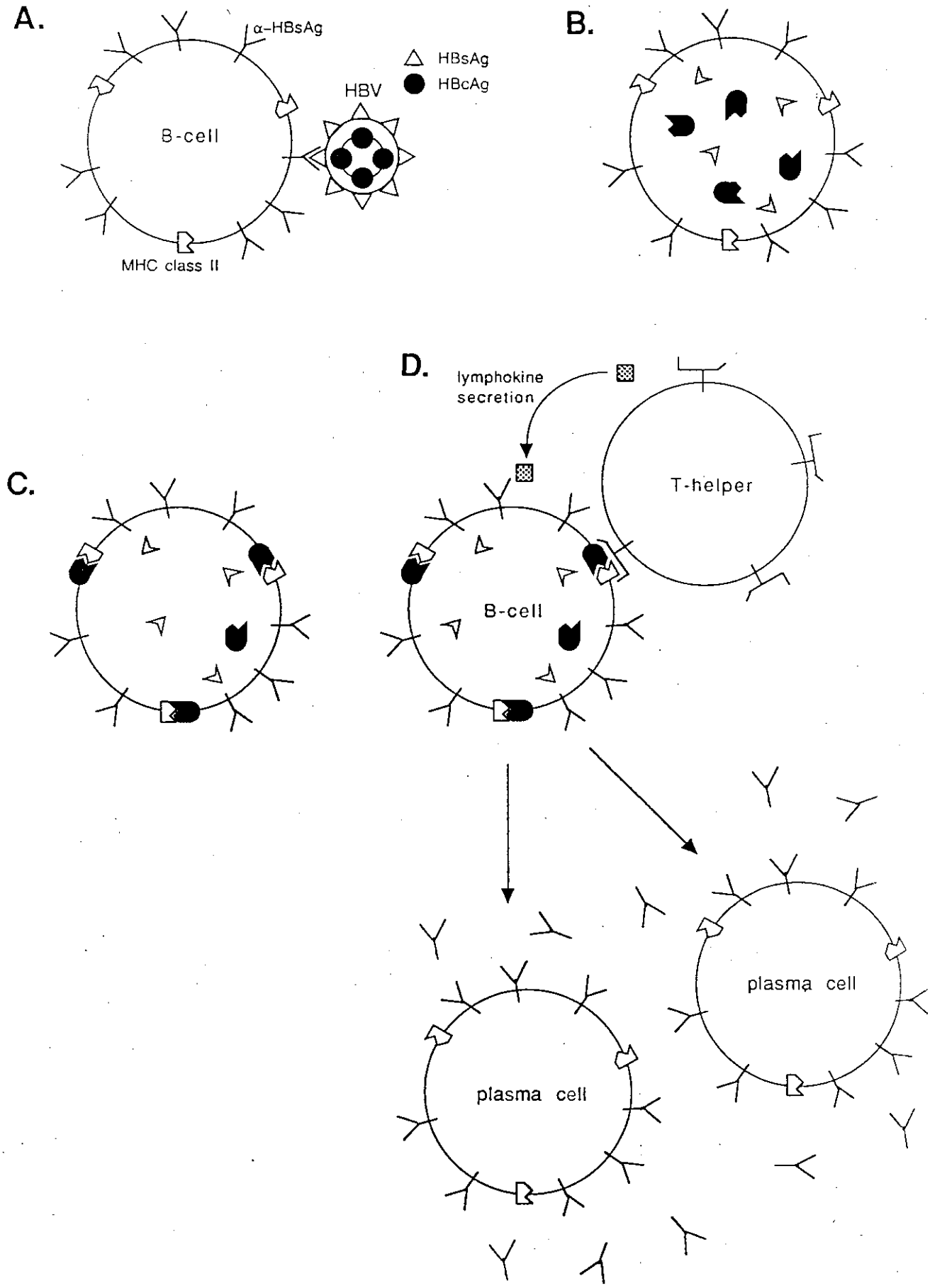
anti-HBcAg antibodies are produced in virtually 100% of chronic HBV infections (Hoofnagle, 1973) indicating that a non-responder phenotype may not exist for this antigen. Vento *et al.* (1985) showed that HBsAg-positive, chronic liver disease patients who have not mounted an anti-HBsAg antibody response, have T-helper cell sensitisation to HBcAg but not to HBsAg (12/12 patients tested). Studies on the T-helper cell response to HBcAg by Milich and his colleagues were preceded by the report that vaccination of chimpanzees with HBcAg produced in *E. coli* could protect them against subsequent challenge with HBV (Murray *et al.*, 1984).

Following immunisation of mice with HBcAg, proliferation of T-helper cells isolated from the immunised mouse could be stimulated by the antigen *in vitro* (Milich and McLachlan, 1986). The degree of T-helper cell immunogenicity displayed by HBcAg along with its ability to function as a T-cell independent antigen (Milich and McLachlan, 1986) could explain the enhanced humoral response that it elicits compared with HBsAg. In the mouse, T-helper cell epitopes of HBcAg have been mapped to specific peptide stretches, and it has been shown that different inbred strains of mice produce a T-cell response to different epitopes (Milich *et al.*, 1987b). This may be due to the differential interaction of peptides with the variable MHC class II antigens expressed by the different strains, as T-helper cells only recognise antigen in association with these antigens.

Milich *et al.* (1987c) went on to investigate the possibility that T-helper cells specific for HBcAg could help B-cells produce antibodies specific for HBsAg. Mice were primed by immunisation with a synthetic peptide sequence of HBcAg known to contain a T-cell epitope. Upon challenge with HBV, the primed mice produced antibodies to HBsAg in much higher titre than unprimed mice (Milich *et al.*, 1987c). This result is consistent with that of Lanzavecchia (1985) who showed that B-cells could function as antigen presenting cells to T-helper cells. The events *in vivo* may be envisioned as follows (figure 1.5): Immunisation with the HBcAg peptide causes clonal expansion of T-helper cells with receptors specific for this peptide in association with MHC class II antigen. Upon challenge with virus, a B-cell specific for HBsAg binds to the virion via cell surface immunoglobulin molecules. Endocytosis brings the virion into the B-cell where its constituent parts undergo proteolytic processing, and a proteolytic fragment of HBcAg is expressed on the surface of the B-cell in association with MHC class II antigen. This complex is recognised by a T-helper cell with a receptor specific for the complex of HBcAg and MHC class II antigen, and the T-helper cell secretes lymphokines onto the B-cell causing its clonal expansion. That is, a T-helper cell specific for HBcAg stimulates

FIGURE 1.5 Model for the production of antibodies to HBsAg by priming T-helper cells specific for HBcAg.

Immunisation with HBcAg or with a peptide representing part of the HBcAg primary sequence causes clonal expansion of T-helper cells with receptors specific for a proteolytic fragment of HBcAg in association with MHC class II antigen. Upon challenge of the immune system with intact virus (A), a B-cell present in the host's immune repertoire and expressing surface immunoglobulin specific for HBsAg (α-HBsAg) binds to the envelope of the virus particle. B) Endocytosis brings the virion into the B-cell where its constituent proteins are subjected to proteolytic processing. C) A proteolytic fragment of HBcAg is expressed on the surface of the B-cell in association with MHC class II antigen. D) The HBcAg/MHC complex is recognised by a T-helper cell with an HBcAg-specific receptor that is present in the blood at a higher concentration than normal due to priming with HBcAg. The T-cell secretes lymphokines onto the B-cell causing its clonal expansion and maturation into a plasma cell secreting anti-HBsAg antibodies.


A.
α-HBsAg
HBsAg
HBcAg
HBV
B-cell
MHC class II
B.
C.
D.
lymphokine
secretion
T-helper
B-cell
plasma cell
plasma cell

the clonal expansion of a B-cell producing antibodies specific for HBsAg.

Because proteolytic processing of internalised antigen is a key step in stimulation of B-cells specific for one component of a particle by T-helper cells specific for another component, the same principle should hold for separate components of the same polypeptide. This hypothesis was confirmed by Milich *et al.* (1988) who showed that a synthetic HBsAg peptide, which did not elicit an antibody response in mice was rendered immunogenic upon linkage to a synthetic T-helper cell epitope from HBcAg. Understanding this system has prompted the expression of chimeric HBcAg particles in *E. coli* in which the HBcAg coding sequence (or part of it) is fused to a heterologous sequence in order to enhance the antibody response against the heterologous polypeptide segment (Clarke *et al.*, 1987; Borisova *et al.*, 1989; Stahl and Murray, 1989; Francis *et al.*, 1990).

HBcAg produced in *E. coli* spontaneously forms particles whose structure appears identical to HBcAg purified from HBV infected liver when observed by electron microscopy (Cohen and Richmond, 1982). The *E. coli* derived HBcAg particles are immunogenic, eliciting an antibody response cross-reactive with HBcAg derived from human liver (Pasek *et al.*, 1979) and antigenic, reacting with anti-HBcAg antibodies in serum from HBV infected individuals (Stahl *et al.*, 1982). Several studies have begun to decipher the features of HBcAg primary sequence required for particle formation.

Stahl *et al.* (1982) synthesised HBcAg in *E. coli* as a fusion protein with the first eight amino acid residues of β-galactosidase fused in frame to HBcAg containing short deletions of amino-terminal residues. Extracts from cells expressing a fusion protein in which the first two amino-terminal residues of HBcAg were deleted, were 100 times more reactive with anti-HBcAg serum (as assayed by radio-immunoassay) than extracts from cells expressing a fusion protein in which the first four amino-terminal residues were deleted. It has been confirmed that the same amount of fusion protein was expressed by cells harbouring the different plasmids (F. Stewart, personal communication); therefore, the difference in cross-reactivity with anti-HBcAg was most likely due to an altered secondary structure, although the ability of the fusion proteins to form particles has not been assessed.

HBcAg mutants, deleted at the carboxyl-terminus, were expressed in *E. coli* and assessed for the capacity to form particles by their sedimentation profile in sucrose gradient and by electron microscopy (Birnbaum and Nassal, 1990). HBcAg deleted to amino acid residue 164, 149 or 144 was able to form particles, while deletion to residue

139 or 138 abolished this capacity. Birnbaum and his colleagues note that deletion to residue 149, completely removing the protamine-like sequence, dramatically reduced the non-specific RNA content of the particles as well as the stability of the particles to SDS or acid treatment. The authors speculate that nucleic acid may contribute to the stability of HBcAg particles.

The carboxy-terminal deletion of HBcAg to residue 149 mimics the carboxyl-terminus of e antigen, which is secreted from HBV infected cells as soluble dimer (Neurath *et al.*, 1979). HBeAg that formed particles when expressed in *E. coli* lacked the 10 amino-terminal residues remaining in serum HBeAg after cleavage of 19 amino acid residues from the 29 residue signal sequence encoded by the preC region. This structural feature may account for the lack of particle formation by native HBeAg, although it is more likely that this attribute is due to the different biosynthetic pathways of HBeAg and HBcAg (Birnbaum and Nassal, 1990).

Petit and Pillot (1985) noted that core particles isolated from the cytoplasm of HBV infected hepatocytes contained HBcAg in different oxidation states. A doublet of HBcAg observed by SDS-PAGE under non-reducing conditions was reduced to a single band upon treatment with β-mercaptoethanol suggesting intramolecular disulphide bonds in the HBcAg molecule. The oxidation state of HBcAg and HBeAg (amino acid residues 1-144) produced in *E. coli* was investigated by Gallina *et al.* (1989). Both antigens formed particles as observed by electron microscopy; SDS-PAGE under non-reducing conditions revealed that HBeAg formed a 32 kD dimer while HBcAg did not even enter the gel indicating that additional disulphide bonds in the carboxy-terminal 34 amino acid residues link HBcAg dimers together in the particle structure. Additional evidence for the importance of intermolecular disulphide bonds in the HBcAg particle structure was the inability to dissociate the particles in 8M urea (although HBeAg antigenic determinants were revealed) while HBeAg particles were completely dissociated by this treatment as assessed by sedimentation properties in sucrose gradients.

The ability of sequences located within HBcAg amino acid residues 3-144 to direct particle formation has been exploited for the construction of fusion proteins containing heterologous sequences expressed in particulate form (Stahl and Murray, 1989). Because of their particulate nature, the chimeric proteins were easy to purify, and an antibody response was elicited against the heterologous epitopes when injected into rabbits. The use of this system to express the viral *X*-gene product (HBxAg) will be discussed in Chapter 5.

1.3.3 Polymerase

The polymerase (*pol*) gene of HBV covers 80% of a translational reading frame in the HBV genome from nucleotide positions 407-2902. This ORF is capable of encoding a protein of 832 amino acids or about 93 kD. The endogenous polymerase function of HBV was originally assigned to this ORF due to the similarity in the size of its coding capacity to the DNA polymerase enzymes of bacteria and viruses (Galibert *et al.*, 1979; Pasek *et al.*, 1979). Sequence homology studies comparing the predicted amino acid sequence from the polymerase ORF with reverse transcriptase sequences from retroviruses and cauliflower mosaic virus showed tracts of strong homology indicating functional relatedness (Toh *et al.*, 1983).

The detection of antibodies directed against a product of the polymerase ORF in serum from HBV infected chimpanzees (McGlynn and Murray, 1988) and humans (Stemler *et al.*, 1988) indicates that it is expressed during the viral life-cycle. The requirement of the *pol*-gene product for replication of the HBV genome was demonstrated in one of the first reports of virus production from transiently transfected hepatoma cells in culture (Yaginuma *et al.*, 1987b). Transfection of cells with a mutant HBV genome carrying a premature termination codon in the polymerase ORF resulted in production of core particles that did not contain viral DNA.

Antisera prepared against fragments of the polymerase protein expressed in *E. coli* (Mack *et al.*, 1988) or against synthetic peptides representing the deduced amino acid sequence of a segment of the *pol*-gene product (Bavand *et al.*, 1989) have detected cross-reacting proteins present in HBV particles produced by cultured hepatoma cells. This protein displays reverse transcriptase activity when immobilised within a polyacrylamide gel following PAGE (Bavand and Laub, 1988; Bavand *et al.*, 1989).

Several domains have been identified within the *pol*-gene product. Immunoprecipitation of DHBV DNA by antibodies directed against the amino-terminus of the polymerase polypeptide indicates that the genome-linked protein of DHBV (and, by inference, HBV) is encoded by this region of the *pol*-gene product (Bartenschlager and Schaller, 1988; Bosch *et al.*, 1988). A model for replication of the HBV genome indicates that the genome-linked protein remains covalently attached to the minus strand DNA after priming reverse transcription of this strand from the viral pregenome mRNA (see section 1.5.2). Sequence comparisons originally identified other domains within the polymerase ORF which displayed homology to the DNA polymerase and reverse

transcriptase domain and to the RNase H domain of retroviral *pol*-genes (Toh *et al.*, 1983; Radziwill *et al.*, 1990).

Mutational analysis of the polymerase ORF was carried out in the context of the complete HBV genome, by assessing the endogenous DNA polymerase activity of core particles isolated from the cytoplasm of transiently transfected cells (Radziwill *et al.*, 1990). Mutations in all three domains within this ORF dramatically reduced endogenous polymerase activity. Mutations in the RNase H domain blocked degradation of the RNA component in the hybrid molecule of viral pregenome RNA and minus strand DNA confirming RNase H activity for this domain and indicating that the other domains can function independently for priming and carrying out DNA synthesis. *pol*-gene mutations could be complemented by supplying a wild-type *pol*-gene product in *trans* from an HBV genome with a mutated core ORF indicating that the polymerase polypeptide exists in a diffusible state. Specificity for reverse transcription of the viral pregenome RNA could arise if this activity of the polymerase is activated after the terminal protein domain binds to a specific target sequence in the viral RNA.

The viral pregenome RNA is packaged into the nucleocapsid where reverse transcription occurs (Enders *et al.*, 1987). Indirect evidence for a specific interaction between the *pol*-gene product and the pregenome RNA has been provided by the observation in both the DHBV model system (Hirsch *et al.*, 1990) and HBV (Bartenschlager *et al.*, 1990) that the *pol*-gene product is required for encapsidation of the pregenome RNA. An 85 nucleotide encapsidation signal within the pregenome RNA (nucleotide positions 3134-3182/1-36) is necessary and sufficient for mediating this function (Bartenschlager *et al.*, 1990; Junker-Niepmann *et al.*, 1990).

1.3.4 HBxAg

The *X*-gene was first described as an ORF in the DNA sequence of the HBV genome covering nucleotide positions 2656-3117 (Galibert *et al.*, 1979). This ORF has a coding capacity of 154 amino acid residues to produce a protein of 17 kD. Based on this size, it was initially proposed that the e antigen was the product of this ORF (Galibert *et al.*, 1981), but this hypothesis was disproved by the discovery that HBeAg can be derived from HBcAg (MacKay *et al.*, 1981). The function of the *X*-gene product (HBxAg) in the viral life-cycle is still under intensive investigation.

There is strong evidence that HBxAg is expressed during viral infection and is

immunogenic. Expression of a ~1 kb X-specific mRNA has been detected in mammalian cells transfected with the complete HBV genome (Gough, 1983; Zelent *et al.*, 1987; Koike *et al.*, 1989) or with a fragment of the HBV genome containing the surface and X ORFs (Simonsen and Levinson, 1983; Saito *et al.*, 1986; Siddiqui *et al.*, 1986; Bulla and Siddiqui, 1988, 1989). While *X*-gene specific transcripts have not been detected in HBV infected liver (Cattaneo *et al*, 1984; Will *et al.*, 1987; Su *et al.*, 1989), a predominantly nuclear, non-polyadenylated RNA of 0.65 kb has been observed in WHV infected liver that hybridised specifically to the X region of the WHV genome (Kaneko and Miller, 1988). In support of evidence for expression of an X-specific mRNA, an X promoter has been identified within 200 nucleotides 5´ to the X ORF which can direct transcription of a heterologous reporter gene (Treinin and Laub, 1987; Siddiqui *et al.*, 1987; Wollersheim *et al.*, 1988).

Direct evidence for the ability of the *X*-gene to encode a protein product has been provided by its expression in both prokaryotic (Kay *et al.*, 1985; Elfassi *et al.*, 1986; Meyers *et al.*, 1986; Pugh *et al.*, 1986; Chisaka *et al.*, 1987; Pfaff *et al.*, 1987; Chen *et al.*, 1988; Jameel *et al.*, 1990; Katayama *et al.*, 1989; Stemler *et al.*, 1990; Wu *et al.*, 1990) and eukaryotic (Moriarty *et al.*, 1985; Siddiqui *et al.*, 1987; Koike *et al.*, 1988; Levrero *et al.*, 1990b) systems and by *in vitro* transcription and translation (Pfaff *et al.*, 1987; Lin and Lo, 1989; Wu *et al.*, 1990). These synthetic antigens as well as synthetic peptides representing segments of HBxAg have served as reagents to detect antibodies to HBxAg in the sera of HBV infected individuals (Kay *et al.*, 1985; Moriarty *et al.*, 1985; Elfassi *et al.*, 1986; Meyers *et al.*, 1986; Pfaff *et al.*, 1987; Koike *et al.*, 1988; Weber *et al.*, 1988; Katayama *et al.*, 1989; Levrero *et al.*, 1990b; Stemler *et al.*, 1990). The immunological response to HBxAg is considered in detail in Chapter 3.

Antisera raised against HBxAg expressed in *E. coli* or against synthetic peptides representing segments of HBxAg have been used to detect cross-reacting proteins in several preparations. HBxAg could not be detected in the serum of HBV infected chimpanzees (Weber *et al.*, 1988), either because it is not manifested in the serum during infection or the quantity present is too low to be detected. Pfaff *et al.* (1987) were unable to detect a polypeptide encoded by the X ORF in liver specimens from 24 HBV infected patients. In contrast, analysis by immunoblotting of proteins extracted from the liver of a patient with chronic HBV infection or from a human hepatoma cell line (PLC/PRF/5) containing integrated HBV DNA resulted in the detection of a 28 kD protein that cross-reacted with antiserum raised against a synthetic peptide fragment of HBxAg (Moriarty *et al.*, 1985). This size was larger than predicted from the deduced amino acid sequence

and could not be due to glycosylation of the polypeptide, as the amino acid sequence reveals no available sites (asparagine-X-serine/threonine). The possibility of a fusion protein containing HBxAg fused to a segment of HBcAg or HBsAg was ruled out by the failure of anti-sera specific for HBcAg or HBsAg to cross-react with the 28 kD species. In addition, the protein did not appear to be bound to DNA as treatment of the protein extracts with DNase did not effect its mobility. Moriarty and her colleagues speculate that the 28 kD protein could represent a fusion of HBxAg with cellular sequences encoded at the junction of one of the HBV integration sites in the host chromosomes. The identification of fusion proteins of this sort is discussed further in section 1.6.

Proteins that cross-reacted with anti-HBxAg serum were immunoprecipitated from the medium of human hepatoma cells (HuH7) transiently transfected with the complete HBV genome (Chang *et al.*, 1987). Proteins of 17 kD, corresponding to the predicted size of HBxAg, and 22 kD were detected by this procedure. Chang and his colleagues speculate that the larger polypeptide could be a cellular protein complexed to HBxAg and co-precipitated with it, or, as above, it could be a fusion protein of HBxAg and cellular sequences. The metabolic labelling of cells and immunoprecipitation procedure were carried out 10-14 days post-transfection by which time input DNA may have integrated into the chromosomes of transfected cells potentially fusing the X ORF to cellular sequences.

HBxAg has been detected by indirect immunofluorescence analysis or immunohistochemical staining of hepatocytes from HBV infected liver (Vitvitski *et al.*, 1988; Katayama *et al.*, 1989), hepatocytes from transgenic mice harbouring the X ORF in their chromatin (Lee *et al.*, 1990), hepatoma cell lines containing integrated HBV DNA (Chisaka *et al.*, 1987), cell lines transfected with the X ORF under control of a heterologous promoter (Siddiqui *et al.*, 1987; Hu *et al.*, 1990; Levrero *et al.*, 1990b), and cell lines transfected with the complete HBV genome (Pugh *et al.*, 1986; Vitvitski *et al.*, 1988). There are conflicting reports about the subcellular location of HBxAg in these preparations. While general cytoplasmic staining was observed in HBV infected hepatocytes and HBxAg transgenic mice, an association with the nuclear periphery (Siddiqui *et al.*, 1987; Chisaka *et al.*, 1987; Levrero *et al.*, 1990b) or more generalised nuclear and cytoplasmic staining (Hu *et al.*, 1990) have been observed in cultured cells. The amino acid sequence of HBxAg does not contain a short, highly basic stretch of residues that is typical of many nuclear proteins (section 1.3.2; figure 1.6), but this does not rule out the capacity to enter the nucleus (Dingwall and Laskey, 1986).

FIGURE 1.6 Sequence comparison of HBxAg isolates.

Alignment of X antigen amino acid sequences in single letter code translated from the nucleotide sequence of eight HBV, five WHV, and one GSHV isolates. Numbers represent amino acid position in the HBV*adyw* sequence. Complete identity at a particular position is indicated by *, while positions containing amino acid residues of homologous structure and charge are indicated by •. Completely conserved cysteine residues are indicated above the HBV*adyw* sequence, and every tenth amino acid residue in the HBV*adyw* sequence is marked with a + above it. Sequence alignment was carried out using the program, "Clustal" (Higgins and Sharp, 1988).

References: HBV*adyw*, Pugh *et al.* (1986)
HBV*ayw*, Galibert *et al.* (1979)
HBV*ayw2*, Bichko *et al.* (1985)
HBV*adw*, Ono *et al.* (1983)
HBV*adw2*, Valenzuela *et al.* (1980)
HBV*adr*, Ono *et al.* (1983)
HBV*adr4*, Fujiyama *et al.* (1983)
HBV*ayr*, Okamoto *et al.* (1986)
WHV1, Etiemble *et al.* (1986)
WHV2, Cohen *et al.* (1988)
WHV3, Cohen *et al.* (1988)
WHV4, Girones *et al.* (1989)
WHV5, Galibert *et al.* (1982)
GSHV, Seeger *et al.* (1984)

```
          1                                                          60
                C  +          +          +          +          +          +
HBVadyw   MAARLCCQLDPARDVLCLRPVGAESCGRPFSGPLGALSSSSLPAVPAAHGAHLSLRGLPV
HBVayw    MAARLCCQLDPARDVLCLRPVGAESCGRPFSGSLGTLSSPSPSAVPTDHGAHLSLRGLPV
HBVayw2   MAARLCCQLDPARDVLCLRPVGAESRGRPFSGSLGTLSSPSPSAVSTDHGAHLSLRGLPV
HBVadw    MAARLYCQLDPSRDVLCLRPVGAESRGRPLSGPLGTLSSPSPSAVPADHGAHLSLRGLPV
HBVadw2   MAARLYCQLDPSRDVLCLRPVGAESRGRPLSGPLGTLSSPSPSAVPADHGAHLSLRGLPV
HBVadr    MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGALPSPSSSAVPADHGAHLSLRGLPV
HBVadr4   MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLPSPSSSAVPADHGAHLSLRGLFV
HBVayr    MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPSAVPSDHGAHLSLRGLPV
WHV1      MAARLCCQLDSARDVLLLRPIGPQSSGPPFPRPAAGSAASSASSPSPSDESDLPLGRLPA
WHV2      MAARLCCQLDSARDVLLLRPFGPQSSGPPFPRPAAGSAASSTSSPSPSDESDLPLGRLPA
WHV3      MAARLCCQLDSARDVLLLRPFGPQSSGPSFPRPAAGSAASSASSPSPSDESDLPLGRLPA
WHV4      MAARLCCPLDSARDVLLLRPFGPQSSGPSFPRPAAGSAASSASSPSPSDESDLPLGRLPA
WHV5      MAARLCCQLDPARDVLLLRPFGSQSSGPPFPRPSAGSAASPASSLSASDESDLPLGRLPA
GSHV      MAARLCCQLDSSRDVLLLRPLRGQPSGPSVSGTSAGSPSSAASAFSSGHQADIPVGRLPA
cons.     ****..*.**..**** ***   .. *.. . . .. .... .. .... ..... * .

          61                                                         117
           C       C+            +          +          +          +
HBVadyw   CAFSSAGPCALRFTSA--RRMETTVNAHQILPKVLHKRTLGLSAMSTTDL-EAYFKDCLF
HBVayw    CAFSSAGPCALRFTSA--RRMETTVNAHQILPKVLHKRTLGLSAMSTTDL-EAYFKDCLF
HBVayw2   CAFSSAGPCALRFTSA--RRMETTVKAQPFLPKVLHKRTLGLSVMSTTDL-EAYFKDCLF
HBVadw    CAFSSAGPCALRFTSA--RCMATTVNAHQILPKVLHKRTLGLPAMSTTDL-EAYFKDCVF
HBVadw2   CAFSSAGPCALRFTSA--RCMETTVNAHQILPKVLHKRTLGLPAMSTTDL-EAYFKDCVF
HBVadr    CAFSSAGPCALRFTSA--RRMETTVNAHQVLPKVLHKRTLGLSAMSTTDL-EAYFKDCVF
HBVadr4   CAFSSAGPCALRFTSA--RRMETTVNAHQVLPKVLHKRTLGLSAMSTTDL-EAYFKDCLF
HBVayr    CAFSSAGPCALRFTSA--RRMETTVNAHRNLPKVLHKRTLGLSAMSTTDL-EAYFKDCVF
WHV1      CFASASGPCCLVFTCADLRTMDSTVN----FVSWHAKRQLGMPS---KDLWTPYIKDQLL
WHV2      CFASASGPCCLVFTCADLRTMDSTVN----FVSWHAKRQLGMPS---KDLWTPYIKDQLL
WHV3      CFASASGPCCLVFTCAELRTMDSTVN----FVSWHANRQLGMPS---KDLWTPYIKDQLL
WHV4      CFASASGPCCLVFTCADLRTMDSTVN----FVSWHANRQLGMPS---KDLWTPYIKDQLL
WHV5      CFASASGPCCLVVTCAELRTMDSTVN----FVSWHANRQLGMPS---KDLWTPYIRDQLL
GSHV      CFYSSAGPCCLGFTCADLRTMDSTVN----FVPWHAKRQLGMMQ---KDFWTAYIRDQLL
cons.     *  *..*** *  *.*  * *..**.    .     .* **.     .*. ..*..* ..

          118                                        154
             +          +      C  +          +
HBVadyw   KDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA
HBVayw    KDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA
HBVayw2   KDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA
HBVadw    KDWEELGEEIRLMIFVLGGCRHKLVCAPAPCNFFTSA
HBVadw2   KDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA
HBVadr    KDWEELGEEIRLKVFVLGGCRH---------NFFTSA
HBVadr4   KDWEELGEEIRLKVFVLGGCRHKLVCSPAPCNFFTSA
HBVayr    NEWEELGEEIRLKVFVLGGCRHKLVCSPAPCNFFTSA
WHV1      TKWEEGSIDPRLSIFVLGGCRHK--CMRLL-------
WHV2      TKWEEGSIDPRLSIFVLGGCRHK--CMRLL-------
WHV3      TKWEEGSIDPRLSIFVLGGCRHK--CMRLL-------
WHV4      TKWEEGSIDPRLSIFVLGGCRHK--CMRLL-------
WHV5      TKWEEGSIDPRLSIFVLGGCRHK--CMRLP-------
GSHV      TLWEEGIIDPRLKLFVLGGCRHK--YM----------
cons.     . ***    . ** .********
```

Recently, HBxAg has been detected as a component of the HBV virion (Wu *et al.*, 1990). Virus particles were incubated with [γ-^{32}P]ATP and subsequently dissociated with SDS. A 16.5 kD labelled polypeptide was immunoprecipitated specifically with anti-HBxAg serum. The significance of phosphorylation of HBxAg in the endogenous kinase reaction will be considered in Chapter 5.

The presence of proteins in various HBV preparations that cross-react with antisera specific for HBxAg and the mounting of an humoral immune response against HBxAg during HBV infection indicate that an immunogenic protein is expressed from the X ORF of the HBV genome. This ORF is conserved in all mammalian hepadnaviruses and amino acid sequence comparisons show two strong regions of similarity in all species (Colgrove *et al.*, 1989; personal observations, figure 1.6, amino acid positions 1-20 and 132-140) suggesting a conserved function for the *X*-gene products. In their review, Ganem and Varmus (1987) cite their own unpublished results suggesting that the *X*-gene product is necessary for viral growth in the GSHV system. 0/9 animals exposed to viral genomes bearing a frameshift mutation in the X ORF caused by oligonucleotide insertion developed infection, while 1/3 recipients of a genome containing an insertion in the X ORF that maintained its reading frame became viraemic. The effect of a frameshift mutation at the 3 ´ end of the X ORF (following amino acid 117) was investigated upon transient transfection of HUH 7 cells with the mutant HBV genome (Yaginuma *et al.*, 1987b). No effect was observed on the production of core particles containing viral DNA. However, from the mutant plasmid construction used in this study it was possible that a biologically active *X*-gene product was expressed from a second, intact copy of the X ORF. Subsequent studies with a plasmid construction derived from the one described above, but lacking the intact copy of the X ORF, revealed that the mutation in the X ORF reduced viral transcription in a different hepatoma cell line (HepG2) although it was not essential for production of core particles containing viral DNA (Koike *et al.*, 1989). The effect of the HBxAg mutation on HBV transcription is consistent with numerous reports that have now emerged concerning the transactivating function of HBxAg (see appendix II for references).

Three lines of reasoning led to the hypothesis that HBxAg encodes a transcriptional transactivator. 1) HBV replicates in a manner analogous to retroviruses, and all human retroviruses characterised to date encode polypeptides that activate transcription from the viral long terminal repeat (LTR). 2) HBV infection is correlated with the development of HCC (see section 1.6) and many isolated HCC tissues contain HBV DNA integrated

into the host cell chromosomes. However, no specific integration site has been observed in human HCC suggesting that HBV may play a role in the development of neoplasia through the action of one of its gene products in *trans*. 3) One of the mysteries in the search for the function of the X gene is the absence of a homologous ORF in the avian hepadnaviruses (Mandart *et al.*, 1984; Sprengel *et al.*, 1988). It is possible that these species have a cellular gene performing the function served by the *X*-gene product of the mammalian hepadnaviruses. Investigation of the nucleotide sequence of the X ORF indicated that, like retroviral oncogenes, it has a codon usage preference favoured by eukaryotic cellular genes and not by the genes of viruses that infect eukaryotic cells (Miller and Robinson, 1986). Miller and Robinson (1986) speculate that, like retroviral oncogenes, the *X*-gene may be "recently" acquired from cellular sequences. However, unlike the retroviral sequences, a cellular gene has not been detected that contains enough sequence homology to cross-hybridise with X-specific DNA probes. In addition, HBxAg does not function like retroviral oncogenes that can transform cells *in vitro* and rapidly produce tumours *in vivo*. Miller and Robinson (1986) speculated that HBxAg may have a transactivating function like that encoded by the pX genes of human T-cell lymphotropic virus (HTLV) I and II which also have cellular codon usage, do not have a cellular homologue, and do not behave as oncogenes, yet infection with these viruses is linked to neoplasia.

The potential of HBxAg to activate transcription from a variety of transcriptional control elements has come under rigorous investigation, and the targets that have been described are listed in table 4.1. HBV transcriptional control regions, as well as those from heterologous viruses and certain cellular genes are susceptible to transactivation by this promiscuous HBV gene-product. A detailed consideration of the features of transactivation by HBxAg is given in Chapter 4, while the mechanism by which it may carry out this function is discussed in Chapter 6. Here, I will examine the primary structure of HBxAg for features that have been described as integral to the function of various cellular and viral transcriptional activating proteins.

Transcription factors are polypeptides that bind to specific sequence motifs (~8 bp in length) proximal or distal to a gene and influence its transcription, (see section 6A.1 for more detailed discussion; for reviews see Maniatis *et al.*, 1987; Ptashne, 1988; Johnson and McKnight, 1989; Mitchell and Tjian, 1989). Distinct domains that mediate DNA-binding and trans-acting functions have been identified within transcriptional activating polypeptides.

Several different types of DNA-binding domains have been identified for eukaryotic transcription factors. Although DNA-binding capacity has not been observed for HBxAg (Faktor and Shaul, 1990; Jameel *et al.*, 1990; Twu *et al.*, 1990; Unger and Shaul, 1990; Wu *et al.*, 1990), two reports have noted features in the amino acid sequence of HBxAg that appear similar to two well defined DNA-binding motifs (Lo *et al.*, 1988; Lin and Lo, 1989). "Zinc fingers" are formed by tetrahedral coordination of a Zn^{++} ion to the polypeptide with looping out of intervening amino acid residues, and are encoded by a segment of ~30 residues within the protein (reviewed by Evans and Hollenberg, 1988). Two possible sequence motifs comprise zinc fingers: the C_2H_2 motif, which takes the general form C-X_{2-4}-C-X_3-F-X_5-L-X_2-H-X_{3-4}-H, is part of the transcription factors Sp1 and TFIIIA (among others). The C_x motif is composed of 4-6 cysteine residues separated by variable numbers of amino acid residues, but in each case, two non-adjacent pairs are separated by not more than five residues. The primary sequence of the HBxAg isolate used in these studies contains ten cysteine residues, eight of which are completely conserved among the HBV isolates, and four of which are completely conserved among all mammalian hepadnaviral isolates considered in figure 1.6 (residue numbers 7, 61, 69, 137 in the numbering of the HBV*adyw* isolate). Lo *et al.* (1988) speculate that the cysteine residues of HBxAg may form a zinc finger, however, they are not in the proper consensus configuration for zinc-binding in any of the X antigen sequences in figure 1.6. Even proteins that do contain cysteine residues in the proper configuration may not bind DNA, for example, the adenovirus E1a protein (reviewed by Evans and Hollenberg, 1988).

HBxAg produced by *in vitro* transcription and translation may form dimers, which can be detected by SDS-PAGE under non-reducing conditions, indicating a disulphide linkage (Lin and Lo, 1989). Several eukaryotic transcription factors bind DNA only in dimer conformation, for example C/EBP (CCAAT/enhancer binding protein), CREB (cyclic AMP response element binding protein) and Fos/Jun (components of the transcription factor AP1). Dimerisation of these proteins is not dependent on disulphide bonds, but is mediated by an α-helical array of four leucine residues each separated by six intervening residues, the "leucine zipper" (reviewed by Landschulz *et al.*, 1988b). Lin and Lo (1989) speculate that a potential leucine zipper motif in HBxAg could contribute to dimer formation (leu-9, leu-16, ala-23, leu-30 and leu-37 in the amino acid sequence of the HBV*adyw* isolate). Two observations argue against dimer formation mediated by these residues. 1) α-Helical secondary structure is not predicted for this region (figure 3.5). 2) The motif is not conserved when the sequence of several X antigen isolates is considered

(figure 1.6). While the central alanine residue is conserved in all human X antigen isolates, it is not present in the rodent isolates in which a proline is found in this position. Similarly, the leucine residue at position 37 is present in all human isolates but is replaced by serine in the rodent polypeptides. The leucine residue at position 30 is found in only three of fourteen isolates and is not replaced by a similar residue in the others. Primary sequence homologies to other types of DNA-binding domains that have been defined, for example the basic domain of nuclear factor 1 (NF-1) or the homeodomains, are not observed in HBxAg. Nor is a helix-turn-helix secondary structure, predicted or actually observed for many transcriptional control proteins, strongly predicted for HBxAg (see figure 3.5).

The activator domains of eukaryotic transcription factors are distinct from the DNA-binding domain, but are required for transactivating or transrepressing function. An activator domain can be linked to a heterologous DNA-binding domain creating a chimeric transcription factor with the specificity of the DNA-binding domain (reviewed by Ptashne, 1988; Mitchell and Tjian, 1989). Several types of activator domains have been described: transcription factor Sp1 has two activator domains consisting of ~25% glutamine, while that of NF1 is proline rich. Neither of these motifs appear in the primary sequence of HBxAg. The activator domain of several transcription factors including the glucocorticoid receptor and yeast activators GAL4 and GCN4, consist of stretches of negatively charged residues with a predicted α-helical secondary structure. The region of HBxAg from amino acid positions 107-126 contains eight negatively charged residues, has predicted α-helical secondary structure, and could represent an activator domain. The requirement of this region for HBxAg activity is subject to debate at present, and is discussed in detail in section 4.1.

1.4 Viral Transcription

Upon infection of hepatocytes, cellular enzymes convert the virion DNA into a covalently-closed circular molecule that is found only in the nucleus of infected cells (reviewed by Ganem and Varmus, 1987; see section 1.5.2). This DNA species serves as template for viral transcription which is carried out by host-cell RNA polymerase II and regulated by HBxAg and by cellular transcription factors. The hepatropism of HBV is influenced in part by the regulatory effect of liver-specific protein factors on viral transcription. When considering HBV transcriptional control elements it is important to

bear in mind that each one is also part of at least one open reading frame and alterations to the sequence of these elements among hepadnaviral isolates is therefore subject to evolutionary constraints on their coding capacity as well as on their function in viral transcription.

All transcripts encoding viral proteins are of plus strand polarity, are unspliced, and coterminate at a common polyadenylation site (figure 1.3). The 3´ processing site has been mapped to nucleotide position 40±5 (Cattaneo *et al.*, 1983; Simonsen and Levinson, 1983; Cattaneo *et al.*, 1984). This termination site is ~20 nucleotides downstream of the conserved hexanucleotide 16-TATAAA-21 which is homologous to the consensus sequence located at a similar distance from the 3´ processing site of cellular genes transcribed by RNA polymerase II (Proudfoot and Brownlee, 1976). The evidence for production in low abundance of an X-specific mRNA has been discussed (section 1.3.4). The major HBV transcripts are 3.3 kb mRNAs that are greater than genome-unit length and subgenomic mRNAs of 2.0 kb and 2.3 kb (excluding poly A tails).

1.4.1 The subgenomic mRNAs

The various HBsAg polypeptides are not produced by processing of a polyprotein but by differential transcription of the pre-S and surface ORFs. The 2.3 kb mRNA contains the coding sequence for the preS1, preS2, and surface domains of the large-S polypeptides as well as for the X gene. Translation of this mRNA in *Xenopus* oocytes yielded only large-S products indicating that internal initiation sites are not used (Standring *et al.*, 1986), and this mRNA species is referred to henceforth as the preS1 mRNA.

The initiation site of the preS1 mRNA has been mapped to nucleotide position 907±4 in RNAs extracted from HBV infected liver (Will *et al.*, 1987), PLC/PRF/5 cells (Ou and Rutter, 1985) or HuH7 cells transfected with HBV DNA and producing virus particles (Yaginuma *et al.*, 1987b). This site is 25 nucleotides downstream of the sequence 876-TATATAA-882 which corresponds in position and sequence to the "TATA box" found in most eukaryotic promoters. A minimal preS1 promoter region (nucleotide positions 816-941) has been determined by assessment of the promoter activity of DNA fragments derived from this region and fused to a reporter gene (Raney *et al.*, 1990).

The smaller 2.0 kb subgenomic mRNAs have heterogenous 5´ termini straddling the preS2 initiation codon at position 1272 (Standring *et al.*, 1984; Ou and Rutter, 1985; Siddiqui *et al.*, 1986; Yaginuma *et al.*, 1987b) and are referred to collectively as the

preS2/S mRNAs (figure 1.4). Experiments mapping the HBV transcripts from PLC/PRF/5 cells (Ou and Rutter, 1985) or from HuH7 cells transfected with HBV DNA (Yaginuma *et al.*, 1987b) indicated that approximately half of the preS2/S mRNAs initiate upstream of the preS2 initiation codon to produce a transcript containing both preS2 and surface ORFs, while the other half initiate downstream of this site and encode only the major-S polypeptides. However, as noted above, the major-S species are the predominant form of HBsAg produced by infected hepatocytes. The expression of major-S polypeptides (along with middle-S forms) from mRNA containing the initiation codons of the preS2 and surface domains has been demonstrated following injection of this mRNA into *Xenopus* oocytes (Standring *et al.*, 1986), or expression of this mRNA from a heterologous promoter in mammalian cells (McLachlan *et al.*, 1987; Ou and Rutter, 1987; Molnar-Kimber *et al.*, 1988) indicating inefficient utilisation of the preS2 initiation codon. Consistent with these results is the observation that the nucleotide sequence surrounding the preS2 initiation codon has two mismatches with the optimal translation initiation sequence described by Kozak (1986), while that surrounding the initiation codon of the surface ORF has only one mismatch.

Examination of the DNA sequence upstream of start sites of the preS2/S mRNAs reveals that the promoter does not contain a "TATA" sequence. Detailed mutational analysis of another promoter lacking a "TATA box", the SV40 late promoter, revealed a region from -31 to -21 (relative to the transcription initiation site) that was important in the control of the level of late mRNA expression and for setting its initiation site. (Brady *et al.*, 1982). The DNA sequence upstream of the preS2/S mRNA start sites (nucleotide positions 1222-1233) contains homology to this region of the SV40 late promoter at a similar position relative to initiation of transcription (Cattaneo *et al.*, 1983). The importance of this region for transcription of the preS/S mRNAs is unclear (figure 1.7). Raney *et al.* (1989) showed that a DNA fragment representing nucleotide 1063-1410 could direct maximal transcription of the chloramphenicol acetyl transferase (CAT) reporter gene relative to the level of CAT transcribed when this gene was inserted into the complete HBV genome at position 1310. Deletion of the fragment from the 5′ end to nucleotide 1153 reduced transcriptional activity two-fold, while deletion to nucleotide 1221 (leaving the SV40-homologous region intact) abolished promoter function indicating that sequences upstream of the region of homology were important for promoter activity. Employing a similar approach, De-Medina *et al.* (1988) defined a region from nucleotide positions 1179-1310 that showed maximal promoter activity in differentiated hepatoma

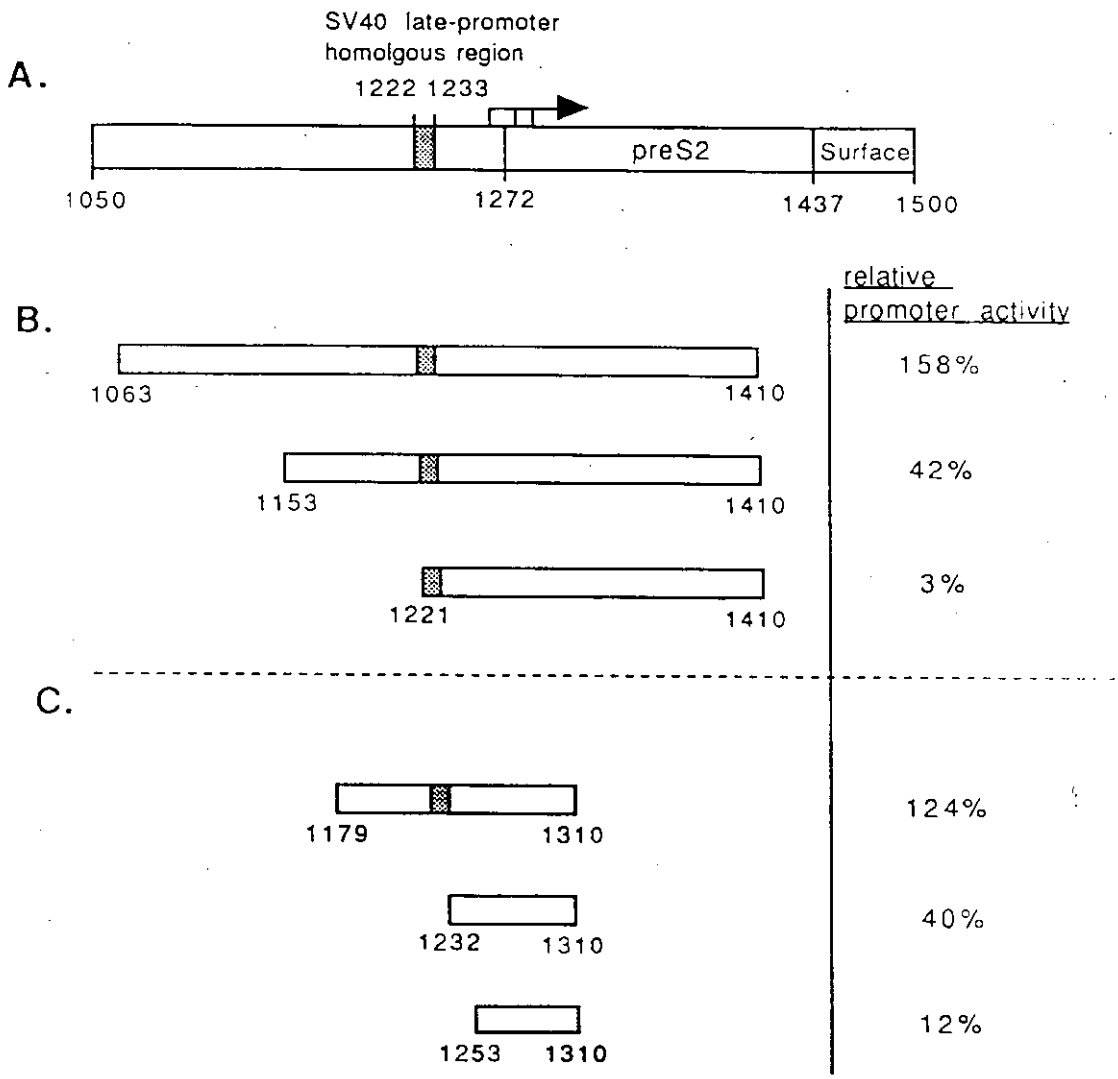


FIGURE 1.7 preS2/S promoter region

A) Representation of HBV nucleotide positions 1050-1500 encompassing the preS2/S promoter region. The region of homology to the SV40 late promoter and the positions of the initiation codons for the preS2 (1272) and surface (1437) ORFs are indicated. The arrow represents the transcriptional initiation sites of the preS2/S mRNAs.

B) Analysis of promoter activity in human hepatoma cells (HepG2) of fragments of the HBV genome fused to CAT (Raney *et al.*, 1989). Promoter activity is given as a percentage of CAT activity relative to that obtained when the CAT gene was inserted into the complete HBV genome at position 1410.

C) Analysis of promoter activity in PLC/PRF/5 cells (De-Medina *et al.*, 1988). Promoter activity is given as a percentage of CAT activity relative to that obtained from a fragment of the HBV genome representing nucleotide positions 1010-1310 fused to CAT.

cells. In this system a promoter region deleted to nucleotide 1232 (removing the SV40-homologous region) retained transcriptional activity, albeit at a level three-fold lower than maximum, while removal of a further 20 nucleotides yielded one-tenth of the original promoter activity indicating that sequences downstream of the region of homology are important for promoter activity.

In vitro binding of protein factors extracted from the nuclei of human hepatoma cells (HepG2) has been identified both within and outwith the SV40-homologous region (Raney *et al.*, 1989). One of the factors binding outwith this region has been identified as nuclear factor 1 (NF-1) (Nagata *et al.*, 1982) which binds to nucleotide positions 1064-1084 (Shaul *et al.*, 1986), although this binding site does not appear to be important for preS2/S promoter activity in differentiated hepatoma cells (De-Medina *et al.*, 1988).

The relative amounts of the various forms of HBsAg are regulated, in part, by the differential activities of the two subgenomic mRNA promoters. The preS2/S promoter displays much higher activity than the preS1 promoter in various systems including: 1) HBV infected liver (Cattaneo *et al.*, 1984), 2) human hepatoma cells transiently (Chang *et al.*, 1987; Yaginuma *et al.*, 1987b) or stably (Sureau *et al.*, 1986) transfected with the complete HBV genome and producing virus, 3) human hepatoma cells transiently transfected with a plasmid construction containing the complete preS1 mRNA transcription unit (Bulla and Siddiqui, 1988,1989; Yuh and Ting, 1990), or 4) the HCC cell line, PLC/PRF/5 (Ou and Rutter, 1985). In the latter system, mRNAs initiating under control of the preS2/S promoter represented 98% of the total surface antigen message. In contrast, the two subgenomic mRNA promoters, isolated from one another and fused to a reporter gene, display similar transcriptional activity (Siddiqui *et al.*, 1986; Antonucci and Rutter, 1989; Bulla and Siddiqui, 1989; Chang and Ting, 1989). Bulla and Siddiqui (1989) have described a negative regulatory region within the preS1 coding region (nucleotide positions 1219-1280), that, when removed, greatly increases transcription from the preS1 promoter upon transient expression in HepG2 cells. In addition to the systems described above, the preS1 promoter shows much lower activity than the preS2/S promoter upon transient transfection of HepG2 cells with a plasmid construction containing a reporter gene inserted into the complete HBV genome immediately downstream of one promoter or the other (Raney *et al.*, 1990). If the negative region described by Bulla and Siddiqui (1989) is functioning in this system, it is doing so at a distance of ~3 kb as opposed to ~300 bp in the native configuration.

The subgenomic mRNA promoters in isolation (De-Medina *et al.*, 1988; Chang and

Ting, 1989) or in the context of the complete HBV genome (Chang and Ting, 1989; Raney *et al.*, 1990) show preferential activity upon transfection into differentiated hepatoma cells compared to undifferentiated hepatoma cells or other cell types. The preferential activity, however, is far more pronounced for the preS1 promoter, and this observation may be reflected by the capacity of non-hepatic cell lines transfected with HBV DNA to secrete spherical HBsAg particles containing middle- and major-S polypeptides but not HBsAg filaments composed of higher quantities of large-S polypeptides (Dubois *et al.*, 1980; Stratowa *et al.*, 1982; Siddiqui, 1983; Stenlund *et al.*, 1983; Wang *et al.*, 1983; Zelent *et al.*, 1987). In addition, analysis of HBV transcripts in the tissues of transgenic mice containing the complete HBV genome incorporated into their chromatin, revealed preS1 mRNA exclusively in the liver while preS2/S mRNAs are found in many other tissues (Araki *et al.*, 1989; Farza *et al.*, 1989).

Protein-binding sites in the preS1 promoter were analysed by DNase I protection (Chang, H.K. *et al.*, 1989; Zhou and Yen, 1991) and by alteration of mobility in acrylamide gel (Zhou and Yen, 1991). Binding sites for the ubiquitous transcription factor Oct-1 (octamer-binding protein 1) and liver-specific factor HNF-1 (hepatocyte nuclear factor 1) have been identified at nucleotide positions 846-853 and 818-829 respectively, which contain strong sequence homology to other known binding sites for these factors. Deletion of either factor binding site yielded reduced transcriptional activity from the preS1 promoter region (Chang, H.K. *et al.*, 1989; Raney *et al.*, 1990; Zhou and Yen, 1991). HNF-1 was identified by virtue of its ability to bind to a common sequence motif in the promoter regions of several liver-specific genes, for example, albumin, α_1-antitrypsin and α-fetoprotein (reviewed by Johnson, 1989). This factor is expressed exclusively in liver cells and its requirement for preS1 promoter activity may contribute to the hepatocyte-specific expression of HBV virion as large-S forms comprise up to 20% of the HBsAg polypeptides in the virion envelope.

1.4.2 Genomic mRNAs

The genomic mRNAs are greater than one genome-unit in length and are produced by read-through of the polyadenylation signal by RNA polymerase II. As with the various forms of HBsAg, the production of core and preC/core polypeptides is regulated by heterogenous initiation of the genomic mRNAs. The predominant 5′ end of genomic mRNAs isolated from HBV infected liver has been mapped to nucleotide position 3100$\pm$2

(Will *et al.*, 1987), which is immediately downstream of the pre-core initiation codon (this transcript will be referred to as core mRNA). Minor, heterogenous initiation sites were mapped upstream of the pre-core start codon (the preC/core mRNAs). The initiation site at nucleotide 3100 was confirmed in mRNA extracted from HuH7 cells transiently transfected with the complete viral genome and producing virus (Yaginuma *et al.*, 1987b), although in this system, the transcripts initiating upstream of the preC start codon represented roughly half of the genomic mRNAs.

As described for the preS2/S mRNAs, no "TATA-box" homology is found within 150 bp upstream of the core mRNA initiation site. The functional core promoter has been identified in the region 2975-3100 by fusion of varying fragments of HBV DNA to reporter genes (Honigwachs *et al.*, 1988; Yaginuma and Koike, 1989; Lopez-Cabrera *et al.*, 1990). The importance of this region for production of virus particles by transiently transfected hepatoma cells was confirmed by Yaginuma and Koike (1989).

The core promoter in isolation (Honigwachs *et al.*, 1988; Lopez-Cabrera *et al.*, 1990) or in the context of the complete HBV genome (Raney *et al.*, 1990) fused to a reporter gene shows preferential activity upon transfection into differentiated hepatoma cells compared to undifferentiated hepatoma cells or other cell types. This may be reflected by the low abundance of genomic mRNAs relative to preS2/S mRNAs in rodent cells transfected with the complete HBV genome in comparison to the relative levels in infected liver tissue (Gough, 1983; Cattaneo *et al.*, 1984). In addition, HBV virion production by transfection of cells with the native HBV genome is limited to differentiated hepatoma cells, however, transcription of the genomic mRNAs under control of the human metallothionein promoter permitted virion production in HeLa cells (Junker *et al.*, 1987).

Liver-specific transcription is attributable, in part, to the binding of liver specific nuclear factors (along with ubiquitous nuclear factors) to the core promoter region described above (Karpen *et al.*, 1988; Yaginuma and Koike, 1989; Lopez-Cabrera *et al.*, 1990). One of these liver-specific factors is C/EBP (discussed in greater detail in Chapter 4) which binds at up to five places in the core promoter and stimulates transcription from this region fused to a reporter gene or in the context of the preC/core coding sequences (Lopez-Cabrera *et al.*, 1990). Stimulation of core promoter activity was only observed with low concentrations of C/EBP, and the authors hypothesize that at low concentration, C/EBP only interacts with particularly high affinity binding sites, while at higher concentrations C/EBP may bind to other sites thereby blocking the binding of other

factors that are important for activating transcription.

The core mRNA encodes the core and polymerase polypeptides, while the preC/core mRNAs encode the HBeAg precursor (Jean-Jean *et al.*, 1989a) and the polymerase polypeptide, although polymerase is less efficiently translated from this mRNA (Ou *et al.*, 1990). Genetic analysis in the DHBV model system indicated that the *pol*-gene product is not produced initially as a core/polymerase fusion protein but by internal initiation of translation (Chang, L.J. *et al.*, 1989; Schlicht *et al.*, 1989b). A mutant DHBV genome containing a stop codon in the core ORF could produce the *pol*-gene product to complement a mutant genome containing a stop codon in the polymerase ORF allowing production of core particles with endogenous polymerase activity. Internal initiation of translation for the *pol*-gene product has also been reported in the HBV system (Jean-Jean *et al.*, 1989b; Roychoudhury and Shih, 1990).

1.4.3 HBV enhancers

Enhancers are segments of DNA that influence the transcriptional activity of a promoter region; they can function from a distant position and in either orientation relative to the promoter. Two regions of the HBV genome have been identified that display enhancer function. The first region that was identified (referred to as the HBV enhancer) is located at nucleotide positions 2300-2550, while a second enhancer region (referred to as HBV enhancer II) has been described more recently and is located in the same region as that identified for the core promoter (nucleotide positions 2950-3100).

1.4.3a The HBV enhancer

The HBV enhancer was initially identified by Shaul *et al.* (1985) who observed increased transcriptional activity from an HBV DNA segment (nucleotide positions 2683-2966) fused to a reporter gene when the sequences 2243-2682 were included in a 5′ position in either orientation. The region of the HBV genome that served as a promoter for transcription in these studies may not be relevant to viral transcription in the infected hepatocyte as it is upstream of the region defined as the core promoter. However, the enhancer region was also able to stimulate transcription when fused to well defined, heterologous promoters including the SV40 early promoter (Shaul *et al.*, 1985; Tognoni *et al.*, 1985; Elfassi, 1987; Vannice and Levinson, 1988; Chang and Ting, 1989;

Honigwachs *et al.*, 1989; Ostapchuk *et al.*, 1989; Patel *et al.*, 1989), the herpes simplex virus thymidine kinase (tk) promoter (Antonucci and Rutter, 1989; Yee, 1989), and the human β-globin promoter (Ben-Levy *et al.*, 1989). Collectively these studies, along with others described below, have defined the HBV enhancer within nucleotide positions 2300-2550.

Transcription from all of the HBV promoters is influenced by the viral enhancer. An HBV DNA fragment encompassing nucleotide positions 2244-2635 fused to the CAT reporter gene directed transcription with heterogenous start sites at positions 2605±15 (Treinin and Laub, 1987) indicating a promoter activity juxtaposed to the X ORF. Transcription from this promoter, however, was influenced by sequences greater than 100 bp upstream from the site of transcription initiation. Deletion of HBV enhancer sequences, 2244-2396, greatly reduced transcriptional activity, while further deletion to 2499 abolished this function indicating that the X promoter is dependent on the presence of enhancer sequences.

Both subgenomic mRNA promoters isolated from one another and fused to a reporter gene are influenced by the viral enhancer (Faktor *et al.*, 1988; De-Medina *et al.*, 1988; Antonucci and Rutter, 1989; Chang and Ting, 1989). In addition, deletion of the enhancer region from a plasmid construction containing the transcription units for both subgenomic mRNAs yielded reduced HBsAg secretion from transiently transfected hepatoma cells compared to that observed with the parent plasmid (Bulla and Siddiqui, 1988; Faktor *et al.*, 1988). Transcription from the core promoter (as defined above) fused to the CAT gene is activated by the presence of the enhancer region (Jameel and Siddiqui, 1986; Antonucci and Rutter, 1989; Honigwachs *et al.*, 1989; Patel *et al.*, 1989). As for HBsAg, secretion of HBeAg from hepatoma cells transiently transfected with a plasmid construction containing the preC/core ORF was higher when the enhancer was present in its native position (Roossinck *et al.*, 1986). The effect of the enhancer on production of all viral transcripts has been confirmed in hepatoma cells transiently transfected with the complete HBV genome and producing virus (Hu and Siddiqui, 1991). Deletion of the enhancer region (2246-2588) reduced initiation of transcription from all of the viral promoters.

The contribution of the enhancer region to the liver-specific expression of viral transcripts in under debate. The following investigations were all carried out with transcriptional control regions fused to a reporter gene, and, in considering this issue, I will only cite studies in which transcription from a plasmid construction containing the

enhancer was directly compared to that from a construction in which the enhancer was region was not present. Patel *et al.*, 1989 noted that the enhancer functioned to increase transcription when fused to the SV40 early promoter upon transfection into differentiated hepatoma cells but not into HeLa cells. Activity exclusively in liver cells was also observed for the enhancer in association with the tk promoter (Yee, 1989) or the preS1 or preS2/S promoters of HBV (Antonucci and Rutter, 1989; Patel *et al.*, 1989). Employing a plasmid construction similar to the HBV enhancer/SV40 early promoter construction used above (Patel *et al.*, 1989), Ostapchuk *et al.* (1989) have observed enhancer activity in HepG2, HeLa and F9 (embryonal carcinoma) cells. This result confirmed other observations of activity in many different cell types of the HBV enhancer in association with the SV40 early promoter (Elfassi *et al.*, 1987; Vannice and Levinson, 1988). Chang and Ting (1989) noted that the enhancer fused to the preS1 promoter showed preferential activity in differentiated hepatocytes compared to poorly differentiated hepatocytes or other cell types. However, this difference was not observed when the enhancer was fused to the preS2/S promoter.

Many factors may contribute to the discrepancy in the results obtained regarding the liver-cell specificity of the HBV enhancer. These include, differences in the DNA fragments used to encompass the enhancer and the placement of the enhancer relative to the initiation site in these manufactured transcriptional control regions. Transient transfection of non-hepatoma cells (HeLa) or poorly differentiated hepatocytes (SkHep1) with the complete HBV genome resulted in reduced secretion of HBsAg and HBeAg compared to differentiated hepatoma cells, HuH7 and HepG2 (Hu and Siddiqui, 1991). However, deletion of the enhancer region (nucleotide positions 2246-2585) resulted in reduced antigen secretion by all cell types suggesting that the liver-cell specificity of the enhancer may be limited.

The regions of the HBV enhancer and upstream sequences that bind nuclear proteins are shown in figure 1.8. The sites originally identified were UE1 (upstream enhancer region 1), UE2, and UE3 which bound liver-specific factors and the E site which bound factors extracted from both liver and HeLa cells (Shaul and Ben-Levy, 1987). The binding of liver-specific protein factors to the UE2 site has been confirmed in an independent study (Patel *et al.*, 1989) although the particular factors that bind to this site and to UE3 have not been identified. The UE1 site binds purified, recombinant, liver-specific C/EBP (Landschulz *et al.*, 1988a) and, in addition, binding of proteins to this site could be efficiently competed by an oligonucleotide comprising an HNF-1 binding site (Patel *et al.*,

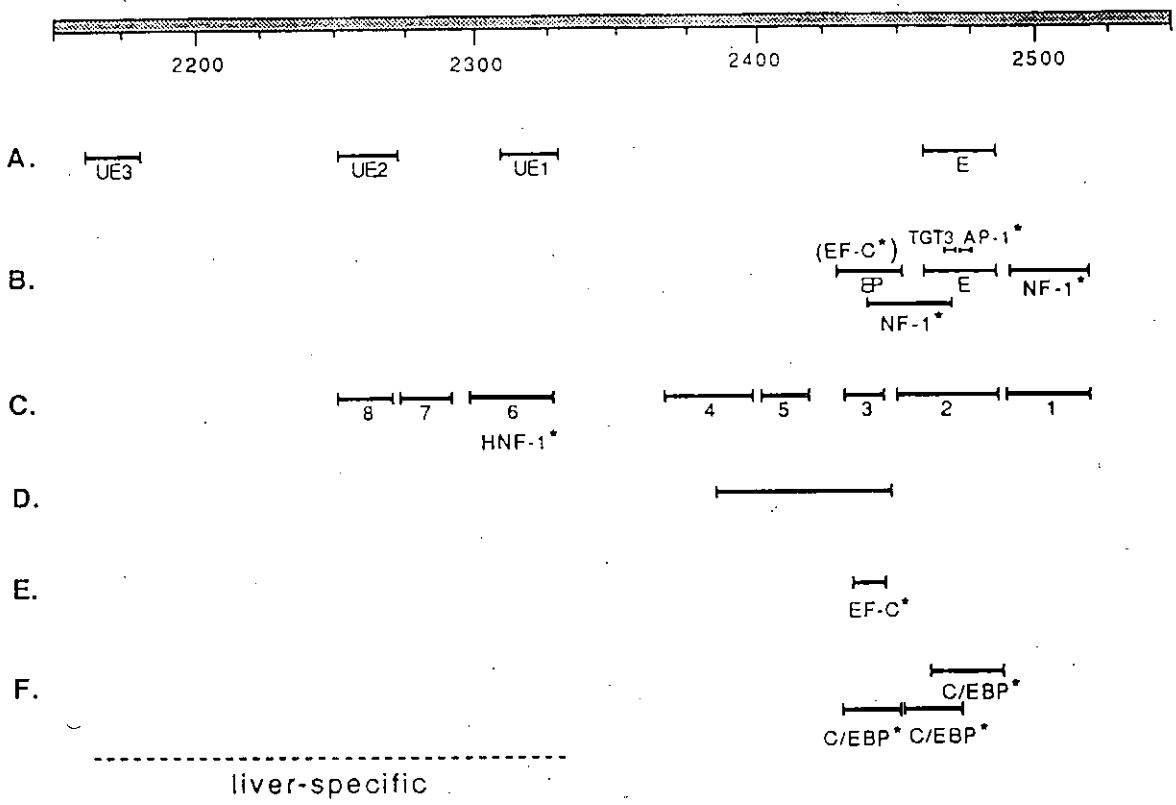


FIGURE 1.8 Nuclear protein binding sites within the HBV enhancer region.

A linear representation of the HBV enhancer (2300-2550) and upstream flanking sequence is shown. Binding regions are indicated by thick lines, and each region is named as designated by the authors. * denotes a site bound by the purified factor that is indicated, or that binding of nuclear proteins to this site was competed by the authentic factor binding site. Sites that bound proteins present only in liver cells are indicated by the dashed line.

References; nucleotide positions of HBV fragments used for binding assays; type of binding assay.

A) Shaul and Ben-Levy (1987); 2107-2654; DNase I protection.
B) Ben-Levy *et al.* (1989); 2395-2654; DNase I protection.
C) Patel *et al.* (1989); 2246-2588; DNase I protection.
D) Karpen *et al.* (1988); 2362-2654; methylation interference.
E) Ostapchuk *et al.* (1989); 2397-2503; DNase I protection.
F) Dikstein *et al.* (1990b); 2395-2515; DNase I protection.

1989).

The E site was initially identified by its ability to bind protein factors in extracts from differentiated hepatocytes (Shaul and Ben-Levy, 1987). Proteins that bind to this site are also present in HeLa cells and other cell types (Shaul and Ben-Levy, 1987; Ben-Levy *et al.*, 1989; Patel *et al.*, 1989). Purified, recombinant, C/EBP binds to this site (Landschulz *et al.*, 1988a; Dikstein *et al.*, 1990b). The proteins present in other cell types that bind to the E site have not been identified, although a polypeptide with DNA-binding properties homologous to C/EBP has been isolated from HeLa cells (Clark *et al.*, 1988). In a chromatography fraction of liver-cell nuclear extract, which lacked a protein binding to the complete E site, a second protein was detected which bound to the DNA sequence 2463-TGTTT-2467 (TGT3) within the E element (Ben-Levy *et al.*, 1989). The TGT3-binding protein was also observed when DNase I protection assays were carried out with a mutated E site that was unable to bind C/EBP (Dikstein *et al.*, 1990a). This motif, together with three 3´ flanking nucleotides are completely conserved in the transcriptional control regions of three genes expressed exclusively in liver cells, α-fetoprotein, α_1-antitrypsin and albumin (Shaul and Ben-Levy, 1989).

The transcription factor AP-1 (activator protein 1) was originally identified as the nuclear protein in HeLa-cell extracts that bound to a sequence motif in the human metallothionein promoter and SV40 enhancer which was known to confer responsiveness to transcriptional activation by tumour promoting phorbol-ester compounds (Lee *et al.*, 1987). AP-1 is functionally constituted by a homodimer of the c-*jun* gene product or a heterodimer of the c-*jun* and c-*fos* gene products. An oligonucleotide comprising an AP-1 binding site efficiently competed for factors binding to an oligonucleotide representing the E-binding site implicating this factor in binding to the E site which contains sequence homology to the AP-1 binding consensus (2470-TGACGCA-2476).

Synthetic multimers of the E site placed downstream of the β-globin transcription unit function to enhance transcription upon transfection of this plasmid construction into hepatoma cells. Cotransfection of cells with this plasmid construction and one expressing c-Jun resulted in increased transcription under control of the β-globin promoter/E-site complex (Faktor *et al.*, 1990). The β-globin promoter alone was not activated by c-Jun, functionally implicating this factor in interacting with the E site. In addition, transcription directed by the β-globin promoter/E-site complex could be stimulated by treatment of transfected cells with phorbol ester (Ben-Levy *et al.*, 1989) providing further evidence for the interaction of AP-1 with this site. The multimerised E site also served as a target for

transactivation by HBxAg (Faktor and Shaul, 1990; see Chapters 4 and 6 for further discussion).

When the complete HBV enhancer was placed downstream of the β-globin transcription unit, a mutation within the E site, which blocked binding to C/EBP (Dikstein *et al.*, 1990b), also blocked enhancer activity (Dikstein *et al.*, 1990a) indicating a functional consequence of C/EBP binding. Transcription from the parent plasmid construction containing an intact enhancer was susceptible to stimulation by C/EBP provided in *trans* at low but not at high concentration (Dikstein *et al.*, 1990b). At high concentrations, recombinant C/EBP binds to additional sites in the enhancer (Dikstein *et al.*, 1990b) (nucleotide positions 2430-2451 and 2451-2470). The mechanism proposed by Lopez-Cabrera and her colleagues, who observed the same phenomenon for activation of transcription by C/EBP from the core promoter could be in effect in this system as well. Binding of proteins to nucleotide positions 2432-2444 could be competed by an oligonucleotide representing DNA sequence from the polyoma virus enhancer which binds the ubiquitous nuclear factor EF-C (Ostapchuk *et al.*, 1989; Ben-Levy *et al.*, 1989). Deletion or mutation of this site dramatically reduced transcriptional activity of the enhancer in association with a heterologous promoter (Ostapchuk *et al.*, 1989; Dikstein *et al.*, 1990a).

The other sites in the HBV enhancer region shown to bind a purified protein are the NF-1 binding sites defined by Ben-Levy *et al.* (1989) (figure 1.8b). Mutation of the downstream NF-1 site reduced transcriptional activity while mutation of the upstream site had little effect (Dikstein *et al.*, 1990a). Patel *et al.* (1989) however, did not detect binding of purified NF-1 to the enhancer, casting confusion on the nature of the protein(s) mediating transcriptional activation at the downstream NF-1 binding site defined by Ben-Levy *et al.* (1989).

The HBV enhancer is unusual in that it is transcribed as part of the mature viral messages for HBsAg and HBcAg and is contained within the coding region for the viral polymerase. Its presence within a mature transcript may be unique to the HBV enhancer among all eukaryotic enhancer regions although, a sequence within the coding region of adenovirus E1a protein shows some enhancer properties (Osborne *et al.*, 1984). The only enhancer property that has been called into question for the HBV enhancer is its orientation independence. Vannice and Levinson (1988) showed that the HBV enhancer functions in only its native orientation when placed in the transcribed region in association with a heterologous promoter. The enhancer is strategically placed between the most

active HBV promoters and affects the production of all viral transcripts. Evidence for binding of protein factors to the enhancer *in vivo* is provided by template competition experiments in which transcription from a plasmid construction containing the enhancer in association with either the core promoter (Karpen *et al.*, 1988) or the preS2/S promoter (this study) is inhibited in hepatoma cells upon cotransfection with a plasmid construction containing the enhancer region. In addition, DNase I hypersensitive sites have been detected in the HBV enhancer region contained in DNA extracted from the liver of HBV transgenic mice indicating the presence of proteins bound to this region (El-Ghor and Burk, 1989; Pourcel *et al.*, 1990).

1.4.3b HBV enhancer II

The core promoter region was defined by Yee (1989) as encompassing nucleotides 2966-3056. This 88 bp fragment (HBV enhancer II) enhanced the transcriptional activity of the tk promoter fused to the CAT gene when placed 5´ or 3´ to the transcription unit and in either orientation. Stimulation of transcription by enhancer II occurred exclusively in differentiated hepatoma cells compared to other cell types. Enhancer II also stimulated transcription from the SV40 early promoter fused to the CAT gene in an orientation and position independent manner, and it could increase transcription from either of the HBV subgenomic mRNA promoters fused to the CAT gene when placed downstream of the transcription unit in its native orientation (Yuh and Ting, 1990). As above, the activity of enhancer II in these studies was specific to differentiated hepatocytes. In contrast to the results obtained with the subgenomic promoters isolated from each other, the enhancer II region activated the preS2/S promoter but not the preS1 promoter when these elements were present in their native configuration (Zhou and Yen, 1990). The mechanism governing preferential activation of a particular subgenomic promoter has yet to be investigated.

1.4.4 Glucocorticoid receptor binding

The glucocorticoid receptor (GR) functions as a transcription factor after binding ligand. GR binding sites have been identified in the transcriptional control regions of a variety of eukaryotic genes including the human growth hormone (Moore *et al.*, 1985) and human metallothionein promoters (Karin *et al.*, 1984) and the mouse mammary tumour

virus LTR (Scheidereit *et al.*, 1983). A binding site for purified rat-liver GR has been identified within the surface ORF at nucleotide positions 1621-1650 (Tur-Kaspa *et al.*, 1988). This site in isolation did not serve as a target for stimulation of transcription by dexamethasone (a glucocorticoid analog), however, a complex of the GR binding site and the HBV enhancer conferred susceptibility to dexamethasone treatment.

1.5 Virus Reproduction

1.5.1 Attachment to hepatocyte

The tissue- and host-specificity of hepadnaviruses is attributed in part to the preS regions of the middle- and large-S polypeptides which can mediate hepatocyte attachment by the HBV virion. The species specificity of individual members of the hepadnavirus family may result from the low level of homology between pre-S regions relative to the other viral ORFs (reviewed by Acs and Price, 1990).

In 1979, Imai *et al.* showed that the gp36 form of HBsAg, which is now known to contain preS2 sequences, could bind gluteraldehyde-polymerised human serum albumin (Gp-HSA) while the p24 and gp27 forms encoded by the surface gene alone could not. A polypeptide fragment of 8 kd can be created by cleavage of gp33 with cyanogen bromide . This fragment contains 55 amino acids derived from the preS2 region and could bind to Gp-HSA but not to polymerised serum albumin from other species (Machida *et al.*, 1984). Additional evidence that the binding of Gp-HSA was mediated by the preS2 region was the inability of HBsAg particles composed exclusively of major-S polypeptides to do so (Persing *et al.*, 1985). Thung and Gerber (1981) showed that only hepatocytes from humans and chimpanzees could bind Gp-HSA on their cell surfaces. Since this binding specificity parallels the host specificity of HBV, a model was proposed for the attachment of HBV to hepatocytes indicating that polymerised serum albumin serves as a bridge between polypeptide sequence in the preS2 region of HBsAg on virus particles and receptors on the surface of hepatocytes.

Several observations have cast doubt on this model (reviewed by Ganem and Varmus,1987). Firstly, polymerised serum albumin is present in sera from patients with liver disease whereas only monomeric forms are present in the circulation of healthy individuals. These forms do not bind HBsAg, and thus how could initial infection of hepatocytes take place? Secondly, naturally polymerised forms of serum albumin do not

bind HBsAg; this property is limited to albumin polymerised with gluteraldehyde (Yu *et al.*, 1985). Thirdly, it has been shown that a 27 amino acid synthetic polypeptide derived from residues 10-36 of the preS1 region can bind directly to hepatocyte receptors, and antiserum raised against this peptide inhibited attachment of hepatoma cells to immobilised HBsAg particles purified from human serum (Neurath *et al.*, 1986b). When recombinant HBsAg particles produced in yeast were examined for the ability to bind liver-cell plasma membranes, only particles containing large-S polypeptides had this capacity (Pontisso *et al.*, 1989), supporting the observation that the preS1 domain can directly mediate binding to hepatocytes.

1.5.2 Genome replication

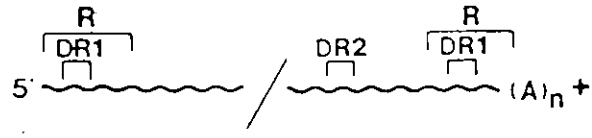
A model for replication of the HBV genome is based on information collected about the replication of HBV itself and of the animal hepadnaviruses. Detailed reviews are provided by Seeger *et al.* (1986), Ganem and Varmus (1987) and Robinson (1990), and figure 1.9 shows a schematic representation of the replication process.

Upon entry of the HBV genome into the nucleus of the infected hepatocyte, the first step in replication is the transition of the genome to a covalently-closed circular, supercoiled DNA molecule. This involves several steps: 1) removal of the oligoribonucleotide from the 5′ end of the plus strand and removal of the protein covalently attached to the 5′ end of the minus strand; 2) trimming of the eight terminally-redundant nucleotides from the 5′ end of the minus strand; 3) completion of the plus strand gap by DNA polymerase; 4) ligation of the 5′ and 3′ ends of the plus strand and of the minus strand; 5) introduction of superhelical turns. The minus strand of the HBV DNA molecule serves as template for transcription of the pregenome RNA, which is greater than one genome-unit in length.

There are several independent pieces of evidence for reverse transcription of the minus strand DNA from this pregenome RNA template. 1) Replication is not semiconservative; the product of replication (the HBV genome) is asymmetric consisting of one long strand and one short strand. 2) Isolated core particles can synthesize minus strand DNA in the presence of the DNA polymerase inhibitor, actinomycin D, and the product is an RNA-DNA hybrid (Summers and Mason, 1982). 3) The deduced amino acid sequence of the polymerase ORF shows homology to known reverse transcriptases (Toh *et al.*, 1983), and a polypeptide with this enzymatic activity is encapsidated in the

FIGURE 1.9 Replication of the HBV genome.

1) A linear representation of the pregenome RNA. (+) Indicates polarity of the HBV sense strand DNA. 2) Protein primed reverse transcription of minus-strand DNA. 3) Degradation of the RNA component in the RNA-DNA hybrid by RNase H, leaving an oligoribonucleotide hybridised to the DR1 sequence at the 3´ end of the minus strand (-). 4) Translocation of the oligoribonucleotide to DR2 at the 5´ end of the minus strand. 5,6) Synthesis of plus strand DNA primed by the oligoribonucleotide. Key to abbreviations and symbols: R = terminal redundancy in the pregenome RNA; DR = direct repeat; $(A)_n$ = polyadenylated 3´ terminus of the pregenome RNA; ● = genome-linked protein; r = eight nucleotide terminal redundancy in minus strand DNA; ∩∪ = oligoribonucleotide. Adapted from Seeger *et al.* (1986).



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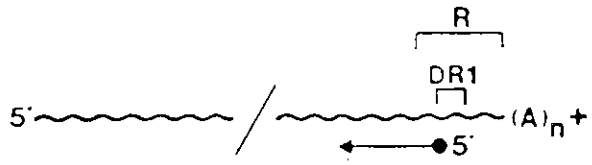

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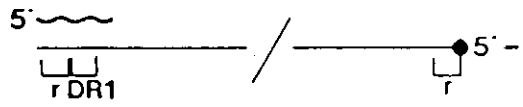

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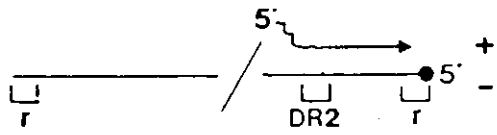

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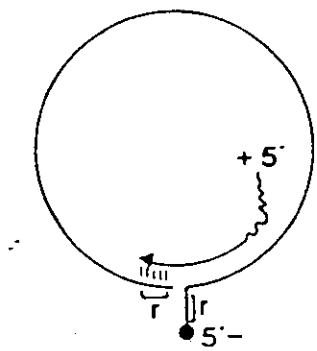

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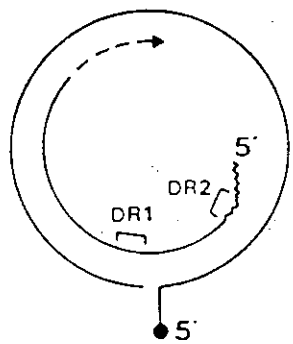

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HBV virion (Bavand and Laub, 1988; Bavand *et al.*, 1989). Pregenome RNA is packaged into viral core particles where it serves as template for reverse transcription of minus strand DNA (Enders *et al.*, 1987). Only core mRNAs and not preC/core mRNAs are packaged as pregenome RNA although both contain the encapsidation signal described above (section 1.3.3). The encapsidation signal is translated in the preC/core mRNAs and the progress of ribosomes through this region may block recognition of the signal (Nassal *et al.*, 1990).

The 5´ end of minus strand DNA has been mapped to nucleotide position 3108 within the DR1 sequence (Will *et al.*, 1987). Viral precedent (reviewed by Wimmer, 1982) indicates that the protein found covalently attached to the 5´ end of the minus strand DNA primes synthesis of that strand. Reverse transcription proceeds along the length of the pregenome mRNA from the DR1 sequence at the 3´ end of the template through to position 3100 which is the 5´ nucleotide of the template. This creates the eight nucleotide terminal redundancy in the minus strand.

The 5´ end of the plus strand DNA has been mapped to nucleotide position 2882 at the 5´ side (in the sense of the minus strand) of the DR2 sequence (Will *et al.*, 1987). The capped oligoribonucleotide that is attached to this end of the strand contains the 11 DR nucleotides, but 6 flanking nucleotides are derived from DR1 (Lien *et al.*, 1986). The following model has been proposed to explain this phenomenon. The RNA component of the RNA-DNA hybrid produced by reverse transcription of the minus strand is degraded by RNase H. A capped oligoribonucleotide derived from the 5´ end of the pregenome mRNA is left hybridised to the 3´ DR1 sequence of the minus strand, and it is subsequently translocated to hybridise with the DR2 sequence at the 5´ end of this strand.

Synthesis of the plus strand is primed by the oligoribonucleotide. The viral DNA polymerase must accomplish a strand transfer at the nick in the minus strand to continue synthesis of the plus strand. This may be facilitated by circularisation of the minus strand mediated by base-pairing of its terminally-redundant nucleotides. Synthesis of the plus strand is constrained in some way as to inhibit its completion which may be due to the concentration of nucleotides available within the nucleocapsid where this synthesis takes place.

1.5.3 Virus assembly

The initial event in assembly of the virus particle is insertion of HBsAg into the membrane of the ER (Patzer *et al.*, 1986; Eble *et al.*, 1987). A model for the conformation of the major-S polypeptide indicates that it contains two transmembrane domains resulting in a loop on the cytoplasmic side of the ER, while both termini are within the lumen of the ER (Eble *et al.*, 1987). Following its association with the ER membrane HBsAg does not become associated with any subsequent intracellular organelle membrane or the plasma membrane (Patzer *et al.*, 1986).

HBcAg forms core particles containing pregenome RNA within the cytoplasm, and the following model has been proposed for association of core particles with HBsAg for formation of the viral envelope (Ou *et al.*, 1986). The pre-C/core polypeptide becomes associated with the ER membrane. Most of this antigen is translocated completely across the membrane where it is processed to HBeAg and secreted, while a small fraction may not get completely translocated but becomes associated with HBsAg that is also embedded in the membrane. The highly basic carboxyl-terminus of the membrane associated preC/core protein may remain on the cytoplasmic side of the ER where it associates with core particles. The core particle then buds into the lumen of the ER, enveloped by the ER membrane and associated HBsAg to form a virus particle. Here, the lipid composition of the envelope is altered from that of the original ER membrane (Eble *et al.*, 1986). Envelope protein is glycosylated in the golgi apparatus, and virus particles are secreted from the cells.

1.6 HBV Infection and Hepatoma

Several lines of evidence indicate a strong correlation between chronic HBV infection and the development of HCC (reviewed by Ganem and Varmus, 1987). 1) The geographical distribution of areas where chronic HBV infection is prevalent coincides with that for high incidence of HCC (Szmuness, 1978). 2) Individuals chronically infected with HBV are subject to more that 100-fold increase in the risk of developing HCC (Beasley, 1981). 3) HBV DNA is found integrated in a high proportion of HCC tumour tissues and in cell lines derived from them.

The latent period between primary HBV infection and the development of HCC is very long (tens of years), and potential causal links between infection and neoplasia that

were initially investigated were derived from examples of slow-transforming, RNA tumour viruses. These viruses, for example avian leukosis viruses and murine leukaemia viruses, activate specific cellular oncogenes (proto-oncogenes) by integration of viral DNA in the flanking sequence in the host chromosome. Extensive analysis of the integrated configuration of HBV DNA in HCC tumour tissues and tumour-derived cell lines has revealed no specific integration site in the host chromosome although particular integration events in or near known proto-oncogenes (or potential proto-oncogenes) have been observed. Dejean *et al.* (1986) described a unique HBV integration event in which the pre-S region of HBV was fused in-frame to a cellular sequence with homology to the v-*erb*-A oncogene, however, altered expression of the fusion gene was not established. A single HBV integration event within a gene homologous to the cyclin A gene has been documented (Wang *et al.*, 1990). Cyclin A is involved in the G_2/M transition of the cell-cycle and the authors speculate that interference with the function of this gene could affect the regulation of cell growth. In the woodchuck model-system, integration of WHV DNA adjacent to c-*myc* (Hsu *et al.*, 1988) or N-*myc* (Fourel *et al.*, 1990) loci, resulting in transcriptional activation of the proto-oncogene, has been observed in a relatively high proportion of HCC tumours, however a similar event is yet to be reported in humans.

Integrated viral DNA may contain deletions and rearrangements, however, junctions between viral and cellular DNA are commonly located in the single stranded gap region of the viral genome (Koshy *et al.*, 1983). While no HBV ORF is universally maintained in HCC tissues, a large portion of the X ORF and upstream sequences are maintained in many cloned integration sites (Dejean *et al.*, 1984; Miyaki *et al.*, 1986; Nagaya *et al.*, 1987; Yaginuma *et al.*, 1987a). This observation led to the hypothesis that HBxAg encoded by integrated HBV sequences may contribute to cellular transformation in a proportion of HCC cases. To test this hypothesis, NIH3T3 cells were stably transfected with the *X*-gene under control of the SV40 early promoter/enhancer complex (Shirakata *et al.*, 1989). Transfected cells that expressed a high amount of X transcript displayed transformed growth characteristics in culture and induced tumours in nude mice. Additional evidence for the neoplastic potential of HBxAg is cited by Ganem (1990); HCC has been induced in two lines of transgenic mice harbouring the *X*-gene under control of its own promoter/enhancer complex, although this effect was not observed for transgenic mice in which HBxAg expression was under control of a heterologous promoter (Lee *et al.*, 1990).

As viral transactivator proteins have oncogenic potential, for example adenovirus E1a, SV40 large T (reviewed by Jones *et al.*, 1988) and HTLV1 $p40^{tax}$ (reviewed by Varmus,

1988), the persistence of transactivating function encoded by integrated HBxAg sequences was examined. A fusion protein of HBxAg (amino acid residues 1-142) and 78 cellular residues encoded by DNA cloned from HCC tissue retained the capacity to transactivate the SV40 early promoter/enhancer complex or the HTLV1 LTR fused to the CAT gene (Wollersheim *et al.*, 1988). The cloned HCC DNA produced a transcript initiating within the X promoter and terminating within the cellular sequence upon transient transfection into hepatoma cells, indicating that the fusion protein could have been expressed in the tumour cells. In addition, the potential importance of HBxAg in sustaining neoplasia was highlighted by conservation of the integrity of this ORF while the surface and polymerase ORFs in the integrated DNA were destroyed by point mutations. The maintenance of transactivating capacity has also been demonstrated for a fusion protein of HBxAg (amino acid residues 1-149) and five cellular residues encoded by a DNA clone isolated from chronic hepatitis tissue (Takada and Koike, 1990). The mechanism by which the transactivating function of HBxAg may contribute to the development of neoplasia is considered in detail in Chapter 6, however, transactivating capacity of proteins encoded by integrated HBV DNA is not limited to the product of the X ORF. HBV DNA and flanking sequence, cloned from an integration site in a hepatoma cell line (Kekule *et al.*, 1990) or HCC tissue (Caselmann *et al.*, 1990), encoded a transcriptional transactivator upon cotransfection into hepatoma cells with a plasmid construction containing the SV40 early promoter/enhancer complex fused to CAT. The sequence of the DNA cloned from the integration sites revealed an ORF encompassing viral sequence fused within the surface ORF to cellular DNA. A frameshift mutation in the preS2 region of this ORF abolished transactivating capacity, while the preS1 region was not essential for this function. Truncated middle-S polypeptides containing as few as 21 amino acid residues of the surface ORF or as many as 139 functioned as transactivators while the full length middle-S polypeptide did not. Interestingly, in the clone derived from HCC tissue, the X ORF was disrupted by a frameshift mutation following amino acid residue 117 which abolished its transactivating capacity suggesting that the function of the middle-S transactivator may be important in maintaining neoplastic growth.

While *cis* or *trans* effects from integrated HBV DNA on transcription of a cellular gene may contribute to the development of HCC, it is also possible that the liver inflammation associated with chronic HBV infection may be sufficient to explain the correlation between HBV infection and hepatoma. The development of HCC has been associated with other inflammatory conditions including schistosomiasis and alcoholic

cirrhosis. All of these conditions result in regenerative hyperplasia increasing the risk of mutation through normal replication processes. An experimental model of this phenomenon has been provided by transgenic mice in which overexpression of the large-S polypeptide leads to accumulation of HBsAg aggregates in the hepatocyte and eventual necrosis (Chisari *et al.*, 1989). The subsequent regenerative hyperplasia has been shown to correlate with the development of HCC in these mice.

A current model indicates that cancer arises through a multistep process resulting in aberration of more than one cellular process (reviewed by Hunter, 1991). Therefore, it is possible that all of the causal associations discussed above can contribute to the cascade of events culminating with the development of HCC.

1.7 Aims of this Thesis

When this research project was initiated in 1986, serological evidence was just emerging for the production of HBxAg during HBV infection. In addition, the importance of the immune response to internal antigens of HBV in mediating protection from subsequent infection had been demonstrated (Murray *et al.*, 1984). The humoral immune response to HBxAg was investigated by expressing fragments of HBxAg in *E. coli* fused to the carboxyl-terminus of β-galactosidase and assessing their reactivity with anti-HBxAg antibodies in the serum of an HBV-infected chimpanzee or of a rabbit inoculated with HBxAg purified from *E. coli* (Chapter 3). In antisera raised against the native antigen, the presence of antibodies directed against particular portions of a molecule can give an indication of its structure when presented to the immune system.

Concurrent with these studies, reports began to emerge that HBxAg could function as a transcriptional transactivator. At the time, all of these experiments had been conducted using target transcriptional control elements (including the HBV enhancer/core promoter or HBV enhancer/X promoter complexes) fused to a heterologous reporter gene. Transactivation of transcription from the preS2/S promoter was suggested by the large quantities of HBsAg secreted from HBV infected cells, and the possibility that this effect was mediated in part by HBxAg was investigated. A system had been established in our laboratory (Jackson, 1987) for the secretion of HBsAg by cultured mouse fibroblasts transiently transfected with a plasmid construction containing the complete transcription unit for the preS2/S mRNAs under control of its own promoter. This expression system was adapted to human hepatoma cells, and the availability of a sensitive

radio-immunoassay for the detection of HBsAg made possible an assessment of the effect of HBxAg on the production of HBsAg when these polypeptides were expressed from a fragment of viral DNA containing these ORFs in their native configuration (Chapter 4). This system was exploited to examine the effect of HBxAg on production of all forms of HBsAg and for a preliminary investigation of the region of the HBV genome that mediates transactivation by HBxAg.

A consideration of the mechanisms by which HBxAg may modulate transcriptional activity (see Chapter 6), and the heretofore unidentified protein kinase activity associated with the HBV virion led to the hypothesis that HBxAg may possess a protein kinase activity. Therefore, kinase activity was assessed for HBxAg produced in *E. coli* as a fusion protein with HBcAg (Chapter 5). The results presented in this thesis along with those published concurrently have begun to elucidate the role for HBxAg in the viral life-cycle.

CHAPTER 2: Materials and Methods

2A MATERIALS

2A.1 Suppliers of Laboratory Reagents

Restriction endonucleases:

Boehringer Mannheim plc; Mannheim, Germany

GIBCO BRL Life Technologies; Paisley, Scotland

New England Biolabs Inc.; Beverly, Massachusetts, U.S.A.

Pharmacia LKB Biotechnology; Milton Keynes, U.K.

E. coli DNA polymerase I (holoenzyme and Klenow fragment), T4 DNA ligase, DNase I (RNase free):

Boehringer Mannheim plc

Thermus aquaticus (*Taq*) DNA polymerase:

International Biotechnologies Inc. (IBI); New Haven, Connecticut, U.S.A.

Deoxynucleoside triphosphates and dideoxynucleoside triphosphates:

Boehringer Mannheim plc

Radioactive nucleoside triphosphates and deoxynucleoside triphosphates:

Amersham International, plc; Aylesbury, U.K.

Standard laboratory reagents (various grades):

BDH Chemicals Ltd.; Poole, U.K.

Fisons Chemicals; Loughborough, U.K.

Gibco BRL Life Technologies

Sigma Chemical Co.; Poole, U.K.

Bacterial media reagents:

Becton-Dickinson U.K. Limited; Oxford, U.K.

Difco Laboratories; East Moseley, U.K.

Reagents for mammalian cell culture:

Gibco BRL Life Technologies

ICN Flow Limited; Rickmansworth, U.K.

Sera-lab; Sussex, U.K.

Sigma Chemical Co.

HBsAg detection kit:

AUSRIA II-125 Diagnostic Kit: Antibody to Hepatitis B Surface Antigen ^{125}I (Human). Abbott Laboratories, Chicago, Illinois, U.S.A.

2A.2 Mammalian Cell Culture

2A.2.1 Mammalian cell lines

Name	Cell type	Source from which cells were obtained	Reference
HepG2	human hepatoma	European Collection of Animal Cell Cultures, Porton Down, U.K.	Knowles *et al.* (1980)
HuH7	human hepatoma	J. Pugh, St. Mary's Hospital, London	Nakabayashi *et al.* (1982)

2A.2.2 Mammalian cell culture media

10 x Glasgow Modification of Eagle's Medium (BHK 21) (McPherson and Stoker, 1962; with modifications by House, Medical Research Council, Institute of Virology, University of Glasgow, Scotland, 1964) was supplied by Gibco BRL Life Technologies and ICN Flow Ltd. 100 x MEM Non-Essential Amino Acids (Eagle, 1959) was supplied by Gibco BRL Life Technologies and ICN Flow Ltd. Foetal bovine serum was supplied by ICN Flow Ltd.

2A.3 Microbiological Materials

2A.3.1 Bacterial strains

Escherichia coli K12 strains used in these studies are listed below:

Strain	Relevant genotype	Use	Reference
ED8654	*sup*E44, *sup*F58, *hsd*R514, *trp*R55, *lac*Y1	general host for plasmid cloning	Borck *et al.* (1976)
GM48	F^-, *dam*3, *dcm*6, *gal*, *ara*, *lac*, *thr*, *leu*, *thi*, *ton*A, *tsx*	*dam* methylation minus host for preparation of plasmid DNA that can be digested with restriction endonucleases sensitive to this type of methylation.	Marinus (1973)
MC1061(λ)	*ara*D139, Δ(*ara*, *leu*)7697, Δ*lac*X74, *gal*U^-, *gal*K^-, *hsr*$^-$, *hsm*$^+$, *str*A, λ^+	contitutive repression of expression from plasmids carrying λPr promoter	MC1061: Casadaban and Cohen (1980); λ^+ derivative: N.E. Murray, unpublished
NF1	K12ΔH1Δ*trp*, *lac*Z$^-$am, λ*Nam*7, *Nam*53, *c*I857ΔH1	heat-inducible expression from plasmids carrying λPr promoter	Stanley and Luzio (1984)
NM522	*hsd*Δ5, Δ(*lac*, *pro*), *sup*E, *thi*, [F′, *pro*A$^+$B$^+$, *lac*ZΔM15, *lac*I^q]	host for bacteriophage M13 vectors and for plasmid pUC8 vector	Gough and Murray (1983)

Strain	Relavant Genotype	Use	Reference
TG1	*hsd*Δ5, Δ(*lac*, *pro*), *sup*E, *thi*, [F´, *tra*D36, *pro*A^+B^+, *lac*ZΔM15, *lac*I^q]	host for bacteriophage M13 vectors and for plasmid pUC8 vector	Amersham International (in catalogue supplied with product, RPN.2322)
BMH71-18 *mut*L	*mut*L::Tn10, Δ(*lac*, *pro*), *sup*E, *thi*, [F´, *pro*A^+B^+, *lac*ZΔM15, *lac*I^q]	tranformation host for heteroduplexes produced by SDM using bacteriophage M13	Kramer *et al.* (1984)
RB791	W3110 [F´, *lac*I^q]	host for expression from a plasmid bearing the *tac* promoter	Brent and Ptashne (1981)

2A.3.2 Bacteriophage and plasmids

E. coli plasmids and bacteriophage used in these studies are listed below. Construction of derivative plasmids and bacteriophage is described in "Results" chapters.

Name	Description	Reference
M13mp8/ M13mp9 M13mp18/ M13mp19	*E. coli* bacteriophage M13 based vectors used for generating single stranded DNA for sequence determination and SDM. These vectors contain a portion of the *lac*Z gene (encoding β-galactosidase) and multiple cloning sites.	Messing and Vieira (1982); Norrander *et al.* (1983)
pUC8	Amp^r. *E. coli* cloning vector containing a portion of the *lac*Z gene (encoding β-galactosidase) and multiple cloning sites.	Vieira and Messing (1982)

Name	Description	Reference
pHBV130	Tet^r. This plasmid contains an HBV DNA insert of greater than 1 genome-unit length, approximately 3.8kb [ca. nucleotides 850-3182/1-1460] in pBR322.	Gough and Murray (1982)
pEX2/3	Amp^r. These plasmids are β-galactosidase fusion protein expression vectors. They contain the coding sequence for a fusion protein of the bacteriophage λ cro protein (amino acids 1-8)/*E. coli* lac repressor (amino acids 318-357)/ β-galactosidase (amino acids 25-1008) under control of the λPr promoter. These vectors contain a polylinker at the 3´ end of the fusion gene which is in a different reading frame in each of the plasmids (pEX2 or pEX3) allowing fusion of further sequences using *in vitro* DNA recombination techniques. In addition each plasmid has the bacteriophage fd transcription termination sequences and stop codons engineered in each translational reading frame downstream of the polylinker.	Stanley and Luzio (1984)
pSV2βG	Amp^r. This plasmid contains the rabbit β-globin coding sequences under control of the SV40 early promoter/enhancer complex.	Gorman *et al.* (1982)
pHBcS111 -156	Amp^r. This plasmid contains the coding sequence for a fusion protein of β-galactosidase (amino acids 1-8)/HBcAg (amino acids 3-144)/major-S polypeptide (amino acids 111-156) under control of the *tac* promoter.	Stahl and Murray (1989)

Name	Description	Reference
pBBX	Amp^r. This plasmid contains the coding sequence for a fusion protein of λ cro protein (amino acids 1-8)/ HBxAg (amino acids 10-154) under control of the λPr promter.	Pugh *et al.* (1986)

2A.3.3 Microbiological media

Luria-broth (L-broth)

10 g Difco Bacto Tryptone
5 g Difco Bacto Yeast extract
10 g NaCl
Made to 1 litre with H_2O, and adjusted to pH 7.2.

L-agar

As L-broth, but containing 15 g agar per litre.

Top-layer agar

10 g trypticase
5 g NaCl
10 g agar
Made to 1 litre with H_2O.

5 x Spizizen salts

2 g $(NH_4)_2SO_4$
14 g K_2HPO_4
6 g KH_2PO_4
1 g tri-sodium citrate
200 mg $MgSO_4$
Made to 1 litre with H_2O

Minimal agar

15 g Difco Bacto agar

200 ml Spizizen salts (5x)

10 ml 20% (w/v) glucose

125 μl vitamin B1 (5 mg/ml)

Made to 1 litre with H_2O

Antibiotics

ampicillin stock solution: 100 mg/ml in H_2O; used at 100 μg/ml

tetracycline stock solution: 100 mg/ml in absolute ethanol; used at 100 μg/ml

2A.4 Solutions

All solutions were prepared in distilled water

20x SSC

3M NaCl

0.3M tri-sodium citrate

10x TBE

0.9M Tris-HCl

0.9M boric acid

2mM EDTA

TE

10mM Tris-HCl pH 8.0

1mM EDTA

Sequencing TE

10mM Tris-HCl pH 8.0

0.1mM EDTA

STE

10mM Tris-HCl pH 8.0

1mM EDTA

150mM NaCl

PBS

1.5mM KH_2PO_4

8mM K_2HPO_4

150mM NaCl

Phenol/chloroform/iso-amyl alcohol (PCI)

25 ml distilled phenol equilibrated with 1M Tris-HCl pH 8.0

24 ml chloroform

1 ml iso-amyl alcohol

2A.5 Antisera

Rabbit 98 anti-HBxAg (Pugh *et al.*, 1986)

Serum from a rabbit inoculated with denatured fusion protein containing amino acids 1-8 of λcro protein fused to the amino terminus of HBxAg (amino acid residues 10-154). The fusion protein was produced in *E. coli* and purified by SDS-polyacrylamide gel electrophoresis as described (Pugh *et al.*, 1986). 30 μg of protein was mixed with Freund's complete adjuvant and injected subcutaneously into the rabbit. A second inoculation with the same components was given after 28 days and a third inoculation of fusion protein without adjuvant was given after a further 28 days. The serum used in these studies was from the third test-bleed withdrawn 8 days after the third inoculation.

Rabbit 87 anti-HBcAg

Serum from a rabbit inoculated with HBcAg produced in *E. coli* (Biogen Inc.). 70 μg of protein was mixed with an equal volume of adjuvant [0.4% Tween 80, 5.0% pluronic L121, 10% squalone, 1 mg/ml muramyl dipeptide in PBS] and injected subcutaneously into the rabbit. A second inoculation with the same components was given after 29 days and a third inoculation with the same components was given after a further 28 days. The serum used in this study was from the third test-bleed withdrawn 9 days after the third incoculation. The adjuvant formulation was suggested by A. Alison, Syntex Inc., Palo Alto, California, U.S.A., who kindly provided the reagents (Allison and Byars, 1986).

Immunoglobulin G (IgG) was purified from this serum by precipitation with ammonium sulphate. Ammonium sulphate was added to the serum to 40%, and the mixture was incubated for 30 minutes at 4°C with stirring. The precipitate was sedimented by centrifugation (12,000 x g) for 10 minutes at 4°C. The pellet was redissolved in a volume of PBS equal to the original volume of serum, and ammonium sulphate was added to 40%. The mixture was incubated for 30 minutes at 4°C with stirring, and the precipitate was sedimented by centrifugation (12,000 x g) for 10 minutes at 4°C. The pellet containing IgG was redissolved in a volume of PBS equal to the original volume of serum and dialyzed against three changes of 1 litre each of PBS.

Chimpanzee Peter serum (Murray *et al.*, 1984)

Serum from chimpanzee Peter from a test-bleed withdrawn 184 days after experimental infection with HBV subtype *ayw*.

Monoclonal antibody MA18/7; anti-preS1 (Heerman *et al.*, 1984)

Monoclonal antibody was produced by fusion of myeloma cells with spleen cells of a BALB/C mouse inoculated with 42 nm HBV particles of subtype *ayw*. This antibody was kindly supplied by W. Gerlich, University of Gottingen, Germany.

Monoclonal antibody Q19/10; anti-preS2 (Heerman *et al.*, 1988)

Monoclonal antibody was produced by fusion of myeloma cells with spleen cells of a BALB/C mouse inoculated with 22 nm HBsAg paritcles, subtype *adw*. This antibody was kindly supplied by W. Gerlich, University of Gottingen, Germany.

2B METHODS

2B.1 Mammalian Cell Culture

2B.1.1 Cell culture

Human hepatoma cells devoid of integrated HBV sequences (HepG2 and HuH7) were grown in 6% CO_2 at 37°C in Glasgow modification of Eagle's medium (GMEM) supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 1 x non-essential amino acids, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% (v/v) foetal bovine serum (FCS).

2B.1.2 Transient transfection of hepatoma cells

The method used for transient transfection of hepatoma cells with plasmid DNA was the DEAE-Dextran/chloroquine method essentially as described by Luthman and Magnusson (1983). 4×10^6 cells were seeded onto 9 cm plates 1 day before transfection. Cells were washed twice in 2 ml of PBS at 37°C, and plasmid DNA was added in 5 ml of a solution containing 200 μg/ml DEAE-Dextran (molecular weight 5×10^6; Sigma) and 50 mM Tris-HCl pH 7.3 in serum-free GMEM. DNA was adsorbed for 5 hours (HuH7 cells) or 6 hours (HepG2 cells) at 37°C at which time 50 μl of 10mM chloroquine diphosphate was added (final concentration, 100 μM). Cells were incubated for a further 2 hours (HepG2) or 3 hours (HuH7) after which the transfection solution was removed, cells were washed twice in 2 ml of PBS at 37°C and were incubated in 15 ml of GMEM supplemented with 10% (v/v) FCS for 3.5-4.5 days.

2B.2 Preparation of Nucleic Acids

2B.2.1 Phenol extraction of nucleic acid solutions

Proteins present in DNA and RNA solutions were removed by extraction with phenol (re-distilled and equilibrated with 1M Tris-HCl pH 8.0) or with phenol/chloroform/iso-amyl alcohol (PCI). An equal volume (unless otherwise stated) of phenol or PCI was added to the solution containing nucleic acid, and the two liquid phases were mixed by vigourous inversion or votexing. The mixture was incubated at room temperature for 5 minutes, and

the liquid phases were remixed as above. The phases were separated by centrifugation for 5 minutes at room temperature in a microcentrifuge (17,000 x g) or bench-top centrifuge (3000 x g) depending on the volume. The upper, aqueous phase containing nucleic acid was recovered.

2B.2.2 Precipitation of nucleic acids with ethanol

Nucleic acids were precipitated from aqueous solutions by addition of one-tenth volume (unless otherwise noted) of 3M sodium acetate pH 5.2, and 2.5 volumes of cold absolute ethanol (-20°C). The mixture was inverted gently several times and incubated at -20°C for several hours or -70°C for 30 minutes. Nucleic acids were sedimented by centrifugation (17,000 x g) in a microcentrifuge or Sorvall centrifuge for 15 minutes at 4°C. The supernatant was discarded, the nucleic acid pellet was dried under vacuum and redissolved in H_2O or TE [10mM Tris-HCl pH 8.0, 1mM EDTA] or sequencing TE [10mM Tris-HCl pH 8.0, 0.1mM EDTA].

2B.2.3 Preparation of RNA from mammalian cells in culture

In order to remove any trace of RNase, all salt solutions and H_2O were treated with 0.1% diethyl pyrocarbonate (DEPC), allowed to stand for several hours at room temperature and autoclaved.

a) Preparation of cytoplasmic RNA

Cell lysis buffer:

10mM Tris-HCl pH 7.4
150mM NaCl
3mM $MgCl_2$
1mM EDTA

The method used to prepare cytoplasmic RNA was essentially that described by S. Heckford (personal communication). The medium was removed from a confluent monolayer of mammalian cells in a 90mm plate and the cells were washed twice in 2 ml

of ice-cold PBS. 2 ml of ice-cold PBS was added to the cell monolayer, cells were scraped with a sterile rubber policeman and removed from the plate with a sterile pasteur pipette. The plate was washed with 2 ml of fresh, ice-cold PBS and this was added to the initial 2 ml suspension. The cells from 2-4 plates of cells transfected with the same amount of the same plasmid DNA were harvested as above and combined. The cells were sedimented from suspension by centrifugation (300 x g) for 5 minutes at 4°C and washed by resuspension in 10 ml of PBS. The cells were sedimented by centrifugation (300 x g) for 5 minutes at 4°C and resuspended in 10 cell volumes of lysis buffer. NP40 was added to 0.5% (v/v), cells were vortexed for 30 seconds and the nuclei were removed by centrifugation (1600 x g) for 15 minutes at 4°C. The supernatant was recovered, SDS was added to 0.5% (w/v) and contaminating proteins and DNA were extracted with an equal volume of phenol at 60°C. The upper, aqueous phase was recovered, and, in order to recover RNA left at the interface of the two liquid phases, an equal volume of TE [10mM Tris-HCl pH 8.0, 1mM EDTA] was added to the organic phase, the phases were mixed by vortexing and separated by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature. The upper, aqueous was recovered and combined with the aqueous phase from the original extraction. Contaminating proteins and DNA were extracted from the combined RNA solutions with an equal volume of phenol at 60°C. The upper, aqueous phase was recovered and remaining traces of phenol were extracted with an equal volume of chloroform/isoamyl alcohol (24/1). The upper, aqueous phase was recovered, and RNA was precipitated with ethanol. The RNA pellet was redissolved in H_2O. RNA was stored in aliquots of approximately 40 μg as an ethanol precipitate at -70°C.

b) Preparation of total cellular RNA

Guanidine hydrochloride (GnHCl) solution:

6M guanidine hydrochloride
10mM DTT
25mM EDTA

RNA extraction buffer:

100mM Tris-HCl pH 9.0
100mM NaCl
1mM EDTA
1% (w/v) SDS

The method used to prepare total cellular RNA was a modification of that described by MacDonald *et al.* (1987). Cells were harvested from confluent monolayers as described above, and the cell pellet was resuspended in 10 ml of GnHCl solution. The cells were lysed by homogenisation and 0.33 ml of 3M potassium acetate pH 5.0 and 5.5 ml of cold absolute ethanol (-20°C) were added. The solution was mixed by gentle inversion, and RNA was precipitated by incubation of the solution for at least 4 hours at -20°C. RNA was sedimented by centrifugation (17,000 x g) for 20 minutes at 0°C, and the RNA pellet was redissolved in 7 ml of GnHCl solution. 0.23 ml of 3M potassium acetate pH 5.0 and 3.6 ml of cold absolute ethanol were added to the solution, and RNA was re-precipitated by incubation of the solution for at least 4 hours at -20°C. RNA was sedimented by centrifugation as above, and the RNA pellet was redissolved in 5 ml of RNA extraction buffer. Contaminating proteins were extracted by adding an equal volume of PCI. The phases were mixed vigorously by vortexing for 5 minutes and separated by centrifugation (3,000 x g) for 5 minutes at room temperature. The upper, aqueous phase was recovered, and RNA was precipitated by adding 0.375 ml of 3M sodium acetate pH 5.2 and 10 ml of cold absolute ethanol. The solution was incubated for at least 4 hours at -20°C and RNA was sedimented by centrifugation (17,000 x g) for 20 minutes at 4°C. The RNA pellet was redissolved in H_2O. RNA was stored in aliquots of approximately 40 μg as an ethanol precipitate at -70°C.

2B.2.4 Preparation of plasmid DNA and bacteriophage M13 replicative form (RF) DNA

a) Small-scale preparation of plasmid DNA

Lysis buffer:

50mM glucose
25mM Tris-HCl pH 8.0
10mM EDTA

Lysis solution (freshly prepared):

200mM NaOH
1% (w/v) SDS

The method used for small-scale preparation of plasmid DNA was a modification of that described by Ish-Horowicz and Burke (1981). 5 ml of L-broth containing the appropriate antibiotic (100 μg/ml) was inoculated with a single colony of *E. coli* habouring the desired plasmid, and cells were grown to stationary phase at 37°C (or at 30°C for cells containing a temperature sensitive mutant gene) with shaking. Cells from 1.5 ml of culture were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 1 minute at room temperature. The cell pellet was resuspended in 100 μl of lysis buffer and incubated for 5 minutes at room temperature. 200 μl of lysis solution was added, the suspension was mixed by gentle inversion and incubated for 5 minutes on ice. 150 μl of 5M potassium acetate pH 5.0 (3M potassium acetate, 2M acetic acid) was added, mixed by gentle inversion and incubated for 5 minutes on ice. Precipitated protein, SDS and chromosomal DNA were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at 4°C. Contaminating proteins in the clarified supernatant were extracted with PCI. The upper, aqueous phase was recovered and nucleic acids were precipitated with ethanol. The nucleic acid pellet was dried under vacuum and redissolved in 50 μl H_2O. 1 μl of RNase A (10 mg/ml; Sigma) was added, and the solution was incubated for 20 minutes at 37°C. Contaminating proteins in the solution were extracted with PCI, the upper, aqueous phase was recovered, and plasmid DNA was precipitated with ethanol. The plasmid DNA pellet was dried under vacuum and redissolved in 50 μl H_2O or TE [10mM Tris-HCl pH 8.0, 1mM EDTA].

b) Large-scale preparation of plasmid DNA

Lysis buffer:

25mM Tris-HCl pH 8.0
10mM EDTA
15% (w/v) sucrose

Lysis solution (freshly prepared):

200mM NaOH
1% (w/v) SDS

The method used for large-scale preparation of plasmid DNA was a scaled-up modification of the method for small-scale plasmid DNA preparation described by Birnboim and Doly (1979). 50 ml of L-broth containing the appropriate antibiotic (100 μg/ml) was inoculated with a single colony of *E. coli* harbouring the desired plasmid and cells were grown to stationary phase at 37°C (or at 30°C for cells containing a temperature sensitive mutant gene) with shaking. Cells were sedimented from suspension by centrifugation (12,000 x g) for 10 minutes at 4°C, and the cell pellet was resuspended in 3.5 ml of lysis buffer. 8 mg of lysozyme (Sigma) dissolved in 0.5 ml of lysis buffer was added, and the suspension was incubated for 30 minutes on ice. 8 ml of lysis solution was added, the suspension was mixed by gentle inversion and incubated for 10 minutes on ice. 5 ml of 3M sodium acetate pH 5.2 was added, mixed by gentle inversion and incubated for 10 minutes on ice. Precipitated protein, SDS and chromosomal DNA were sedimented by centrifugation (17,000 x g) for 40 minutes at 4°C. One-twentieth volume of 3M sodium acetate pH 5.2 was added to the clarified supernatant, and nucleic acids were precipitated by adding 2 volumes of absolute ethanol. The nucleic acid pellet was dried under vacuum and redissolved in 0.5 ml H_2O. 10 μl of RNase A (10 mg/ml; Sigma) was added, and the solution was incubated for 30 minutes at 37°C. Contaminating proteins in the solution were extracted with phenol (1 extraction) and then PCI (2 extractions). The upper, aqueous phase was recovered and plasmid DNA was precipitated with ethanol. The plasmid DNA pellet was dried under vacuum and redissolved in 1 ml H_2O.

c) Preparation of bacteriophage M13 replicative form (RF), double stranded DNA

A fresh, single plaque of M13 was picked with a sterile wooden toothpick and transferred into 2 ml of an L-broth culture of *E. coli* NM522 cells (OD_{650nm} 0.2), and the culture was grown for 4.5 hours at 37°C with shaking. For small-scale preparation of RF DNA, the cells were sedimented from suspension by centrifugation in a microcentrifuge (17,000 x g) for one minute at room temperature and the procedure used was that for small-scale

preparation of plasmid DNA. For large-scale preparation, 50 μl of the 2 ml culture prepared above was added to 50 ml of an L-broth culture of *E. coli* NM522 cells (OD_{650nm} 0.2), and this culture was grown for 4.5 hours at 37°C with shaking. Cells were sedimented from suspension by centrifugation (12000 x g) for 10 minutes at 4°C. From this point onwards the procedure was that of large-scale preparation of plasmid DNA.

2B.2.5 Quantification of nucleic acids

The optical density (OD) of nucleic acid solutions at 260nm was measured using a Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometre. An OD_{260nm} value of 1.0 represents a concentration of 50 μg/ml for DNA and 38 μg/ml for RNA.

2B.2.6 Transformation of *E. coli* with bacteriophage M13 DNA or plasmid DNA

a) Calcium chloride method

This method for transformation of *E. coli* was a modification of that described by Mandel and Higa (1970).

M13 DNA: *E. coli* were made competent for uptake of DNA by the following procedure. 5 ml of L-broth was inoculated with a single colony of *E. coli* NM522, TG1, or BMH71-18 *mut*L and the culture was grown to stationary phase at 37°C with shaking. 100 ml of L-broth was inoculated with 1 ml of the stationary phase culture and cells were grown to OD_{650nm} 0.3 at 37°C with shaking. The cell culture was incubated for 10 minutes on ice, and cells were sedimented from 20 ml of culture by centrifugation (3000 x g) for 5 minutes at 4°C. The cell pellet was resuspended in 10 ml of ice-cold 100mM $CaCl_2$ and incubated for 30 minutes on ice. Cells were sedimented from suspension by centrifugation (3000 x g) for 5 minutes at 4°C, and the cell pellet was resuspended in 2 ml of ice-cold 100mM $CaCl_2$.

For transformation of competent cells, approximately 0.1 ng of RF form or 1 ng of single stranded bacteriophage DNA was added to 200 μl of cell suspension in glass tubes, and the mixture was incubated for 30 minutes on ice. The cells were heat-shocked by incubation for 4 minutes at 42°C and added to 3.5 ml of molten top-layer agar containing:

200 μl of the L-broth culture at OD_{650nm} 0.3 as plating cells, 30 μl X-gal [20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethylformamide] and 20 μl IPTG [20 mg/ml isopropyl-β-D-thiogalactopyranaside in dimethylformamide] for selection of blue versus colourless plaques. Transformed cells were plated on minimal agar plates supplemented with vitamin B1 (final concentration, 0.625 μg/ml). Non-recombinant bacteriophage produce β-galactosidase yielding a blue colour on selective medium while recombinant bacteriophage are incapable of producing β-galactosidase and yield a clear, colourless plaque on selective medium.

Plasmid DNA: Preparation of transformation competent cells was performed as described above except that; 1) cells containing a temperature sensitive mutant gene were grown at 30°C, 2) following dilution of the stationary phase culture, cells other than *E. coli* NM522 or TG1 were grown to OD_{650nm} 0.5 and 3) the final cell suspension in 2 ml 100mM $CaCl_2$ was incubated for a further 30 minutes on ice instead of being used immediately.

For transformation of competent cells, approximately 0.1 ng of plasmid DNA was added to 50 μl of cell suspension and the mixture was incubated on ice for 30 minutes. The cells were heat-shocked by incubation for 4 minutes at 42°C after which 200 μl of fresh L-broth was added. The cells were shaken for 1 hour at the appropriate temperature to allow expression of the antibiotic gene carried by the plasmid. Transformed cells were plated on L-agar plates containing 100 μg/ml of the appropriate antibiotic. Insertion of heterologous DNA into the polylinker in plasmid pUC8 was detected by colour selection on L-agar plates containing 100 μg/ml ampicillin, 40 μg/ml X-gal and 40 μg/ml IPTG.

b). DMSO method

Transformation buffer:

10% (w/v) polyethylene glycol, molecular weight 3000 (Sigma)
5% (v/v) dimethylsulphoxide
10mM $MgCl_2$
10mM $MgSO_4$
in L-broth

This method for transformation of *E. coli* was essentially that described by Chung and Miller (1988). Cells were grown to the same OD_{650nm} as for the $CaCl_2$ method, incubated for 10 minutes on ice, and sedimented from 20 ml of culture by centrifugation (3000 x g) for 5 minutes at 4°C. The cell pellet was resuspended in 2 ml of transformation buffer, and incubated for 30 minutes on ice. 0.1 ng of plasmid DNA was added to 100 μl of cell suspension, and the mixture was incubated for 30 minutes on ice. For bacteriophage M13, transformed cells were plated immediately as above. For plasmid DNA, 200 μl of 20mM glucose in transformation buffer was added to the transformed cells, cells were shaken for 1 hour at the appropriate temperature and plated as above.

2B.3 Enzymatic Manipulation of DNA

2B.3.1 Digestion of DNA with restriction endonucleases

DNA was digested with approximately 5U of restriction endonuclease per μg of DNA using buffer and temperature conditions recommended by the manufacuterer. Digestion reactions were terminated by heating for 10 minutes at 65°C, by phenol extraction, or by addition of DNA sample buffer for agarose gel electrophoresis (section 2B.4.1).

2B.3.2 Filling-in 3´ recessed termini of DNA

The 3´ recessed termini created by digestion of DNA with certain restriction endonulceases were rendered blunt by the action of *E. coli* DNA polymerase I (Klenow fragment). 10 μg of plasmid DNA was digested with the appropriate restriction endonuclease, the reaction mixture was extracted with phenol, and cleaved DNA was precipitated with ethanol. The DNA pellet was dried under vacuum, redissolved in 33 μl H_2O, and incubated for 1 hour at 37°C with the following:

4 μl 100mM Tris-HCl pH 8.0, 100mM $MgCl_2$
1 μl 10mM each dGTP, dATP, dTTP, dCTP
2 μl *E. coli* DNA polymerase I (Klenow fragment) (5 U/μl; Boehringer)

The reaction was terminated either by phenol extraction or by addition of DNA sample buffer for agarose gel electrophoresis (section 2B.4.1).

2B.3.3 *In vitro* recombination of DNA fragments

10 x ligation buffer (for either blunt or cohesive ends):

0.5M Tris-HCl pH 7.4
0.1M $MgCl_2$
0.2M DTT
10mM ATP

Linear DNA fragments were recombined *in vitro* by the action of bacteriophage T4 DNA ligase (Boehringer). Ligation reactions were carried out for 16 hours at 12°C in the following 10 μl reaction mixture:

7 μl desired insert DNA in H_2O (300 ng in total)
1 μl desired, linearised, plasmid DNA in H_2O (100 ng/μl)
1 μl 10 x ligation buffer
1 μl T4 DNA ligase (1 U/μl; Boehringer)

2B.4 Electrophoresis of Nucleic Acids

2B.4.1 Electrophoresis of DNA in agarose gels

5 x DNA sample buffer:

15% (w/v) ficoll, molecular weight 400,000
50 mM EDTA
0.125% (w/v) bromophenol blue
0.125% (w/v) xylene cyanol FF

DNA was fractionated on the basis of fragment size by electrophoresis in submerged, horizontal, agarose, slab gels. Agarose gels consisted of 0.6% to 1.5% (w/v) agarose (depending on the size of the DNA fragments to be separated) melted in 1 x TBE buffer in a microwave oven. Gels were cast containing 0.5 μg/ml ethidium bromide. Prior to loading, 0.2 volumes of 5 x DNA sample buffer were added to each sample.

Electrophoresis was carried out in 1 x TBE buffer, at 5-20 volts(V)/cm. 1 kb ladder (Bethesda Research Laboratories) was used for DNA size standards.

2B.4.1a Gel photography

Nucleic acids stained with ethidium bromide were visualised under short-wave ultraviolet light using a Cromato-vue UV-transilluminator (302 nm wavelenght). Photographs of gels were taken on a Kodak specialist 3 camera containing a red (A1) filter at f/4.5 for 20 seconds using Ilford HP5 film (5 X 4 inch sheet). The exposed film was developed for 5 minutes at room temperature in Ilford microphen developer. The development reaction was stopped by incubation in 3% acetic acid for 30 seconds, and the image was fixed in Hypam fixer for 5 minutes. The film was washed in cold water and air dried.

2B.4.2 Recovery of DNA from low-melting-temperature agarose gels

The method used to recover DNA from low-melting-temperature agarose gels was essentially that of Wieslander (1979). DNA fragments were fractionated as described above except that low-melting-temperature agarose (Bethesda Research Labortories) was used when casting gels. DNA was visualised under short-wave ultraviolet light, the desired band was excised using at sterile scalpel, and the piece of agarose gel was placed in a 1.5 ml microcentrifuge tube. The gel piece was melted at 65°C for 10 minutes and an equal volume of TE pre-warmed to 37°C was added. The solution was mixed by votexing and placed at 65°C for an additional 5 minutes. The solution was cooled to 37°C and an equal volume of phenol pre-warmed to 37°C was added. The phases were mixed by votexing and immediately separated by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature. The upper, aqueous phase was recovered and re-extracted with phenol pre-warmed to 37°C. The upper, aqueous phase was again recovered, and ethidium bromide and trace amounts of phenol were extracted with an equal volume of butan-1-ol. The lower, aqueous phase was recovered and DNA was precipitated with ethanol. The DNA pellet was dried under vacuum, redissolved in 10 μl H_2O and stored at -20°C.

2B.4.3 Electrophoresis of RNA in agarose gels

10 x MOPS buffer

200mM 3-(*N*-morpholino) propane-sulphonic acid (MOPS)
50mM sodium acetate
10mM EDTA
to pH 7.0 with NaOH

Formamide sample buffer

100 μl 10 x MOPS buffer
200 μl formamide
120 μl formaldehyde (37% (w/v))

Ficoll-dye-EDTA (FDE)

500 μl 0.2M EDTA pH 7.0
0.3 g ficoll, molecular weight 400,000
10 mg bromophenol blue
to 1 ml with H_2O

RNA was fractionated on the basis of size by eletrophoresis in submerged, horizontal, agarose, slab gels containing formaldehyde. The method used was a modification of that described by Lehrach *et al.* (1977). 1% (w/v) agarose gels containing 0.27M formaldehyde were prepared as follows: 1 g of agarose was melted in 88 ml H_2O in a microwave oven. The solution was cooled to 60°C and 10 ml of 10 x MOPS buffer, 2ml of formaldehyde (37% (w/v)) and 10 μl of ethidium bromide (5 mg/ml) were added. 1% (w/v) agarose gels containing 0.675M formaldehyde were prepared as follows: 1 g of agarose was melted in 85ml H_2O in microwave oven. The solution was cooled to 60°C and 10 ml of 10 x MOPS buffer, 5 ml of formaldehyde (37% (w/v)) and 10 μl of ethidium bromide (5 mg/ml) were added. The gel was cast on a horizontal bed.

RNA was prepared from monolayers of mammalian cells in culture and stored in aliquots

of approximately 40 μg at -70°C as an ethanol precipitate as described above (section 2B.2.3). RNA was sedimented by centrifugation (17,000 x g) in a microcentrifuge for 15 minutes at 4°C, and the supernatant was removed carefully by aspiration with a drawn-out, glass capillary tube. The RNA pellet was redissolved in 45 μl H_2O and 5 μl of 10 x DNaseI buffer [1M sodium acetate pH 5.2, 100 mM $MgCl_2$] was added. 1 μl of DNaseI (23 U/μl, RNase free; Boehringer) was added to the RNA solution and it was incubated for 30 minutes at 37°C. 25 μl of DNase treated RNA was added to 25 μl of FDE and the solution was heated to 60°C for 5 minutes. The denatured RNA was snap-cooled on ice, and 10 μl of formamide sample buffer was added.

Electrophoresis of RNA samples was carried out in 1 x MOPS buffer at 5-20 volts/cm until the desired fractionation was achieved. RNA was visualised under short-wave ultraviolet light and photographed as described above (section 2B.4.1a).

2B.5 Radio-Labelling DNA

2B.5.1 Labelling DNA by random priming with hexadeoxyribonucleotide primers

Oligo-labelling buffer (OLB) was prepared as follows:

Solution O:

1.25M Tris-HCl pH 8.0

125mM $MgCl_2$

Solution A:

1.0 ml solution O

18 μl β-mercaptoethanol (14M)

5 μl 100mM dATP

5 μl 100mM dGTP

5 μl 100mM dTTP

Solution B:

2.0M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), adjusted to pH 6.6 with 4M NaOH

Solution C:

hexadeoxyribonucleotides (Pharmacia) suspended at 90 OD units/ml

Solution OLB:

2 parts solution A

5 parts solution B

3 parts solution C

Stored in 10μl aliquots at -20°C.

The method used to label DNA was that of Feinberg and Vogelstein (1983). Plasmid DNA was digested with the appropriate restriction endonuclease and cleavage products were fractionated by electrophoresis in a low-melting-temperature agarose gel. The desired DNA fragment was excised from the gel with a sterile scalpel, and the piece of agarose gel was placed into a preweighed, 1.5 ml microcentrifuge tube. H_2O was added at a ratio of 3 ml H_2O per gram of gel. The tube was incubated at 100°C for 10 minutes to melt the gel and denature the DNA. The DNA fragment in agarose was stored at -20°C, and prior to labelling was re-boiled for 5 minutes and then incubated at 37°C for 10 minutes. Labelling reactions were carried out for 2 hours at 37°C or for 16 hours at room temperature in the following reaction mixture:

35 μl DNA fragment in melted agarose (approx. 10 ng in total)
10 μl OLB
2 μl bovine serum albumin (10 mg/ml)
5 μl [α-^{32}P]dCTP (10 μCi/μl; Amersham)

The labelling reaction was stopped and salt concentration was increased for gel filtration by adding 50 μl of STE [10mM Tris-HCl pH 8.0, 1mM EDTA, 150mM NaCl] and unincorporated radio-labelled nucleotides were removed from the reaction mixture by gel filtration through Sephadex G-50 (Pharmacia) (see below).

2B.5.2 Sephadex gel filtration of DNA (Spun columns)

The method for preparation and use of spun columns was essentially that described by Maniatis *et al.* (1982). Dry Sephadex G-50 beads (Pharmacia) were swollen in an excess of TE [10mM Tris-HCl pH 8.0, 1mM EDTA] overnight at 4°C. Columns were prepared in 1 ml plastic syringes from which the plunger had been removed and the hole plugged with glass wool. Sephadex G-50 was added until the bed filled the whole syringe, and the

column was packed by centrifugation (300 x g) for 3 minutes at room temperature yeilding a bed volume of approximately 0.9 ml. The column was equilibrated by adding 0.5 ml of STE [10mM Tris-HCl pH 8.0, 1mM EDTA, 150mM NaCl] followed by centrifugation (300 x g) for 3 minutes at room temperature. The equilibration step was repeated and the sample added to the column. Unincorporated [α-^{32}P]dCTP was trapped in the column, and labelled DNA was collected by centrifugation (300 x g) for 5 minutes at room temperature.

2B.6 Nucleic Acid Hybridisation

Denaturation solution:

0.5M NaOH
1.5M NaCl

Neutralisation buffer:

1M Tris-HCl pH 8.0
1.5M NaCl

2B.6.1 Transfer of DNA from *E. coli* colonies to membranes (colony lifts)

The method used for colony lifts was essentially that described by Grunstein and Hogness (1975). *E. coli* were transformed with plasmid DNA encoding an antibiotic resistance gene and which had been manipulated *in vitro* to contain inserts of heterologous DNA. Transformed cells were plated on L-agar plates containing the appropriate antibiotic (100 μg/ml) for selection of transformed cells. Resulting colonies were picked individually using sterile wooden toothpicks and transferred in a grid pattern onto a fresh L-agar plate containing the appropriate antibiotic (100 μg/ml). Colonies were allowed to grow overnight at the appropriate temperature. The plates were cooled to 4°C, and the colonies were lifted onto nitrocellulose membrane disks (0.45 μm pore size; Sartorius) by carefully laying the disk onto the plate, marking its position and removing it as soon as it had become moistened. Cells were lysed and DNA denatured by placing the membranes colony side up on blotting paper saturated with denaturation solution for 5

minutes at room temperature. The pH of the membranes was neutralised by placing them colony side up on blotting paper saturated with neutralisation buffer for 5 minutes at room temperature. The neutralisation step was repeated, the membranes were dried on blotting paper and baked in a vacuum oven for 2 hours at 80°C.

2B.6.2 Transfer of DNA from bacteriophage M13 plaques to membranes (plaque lifts)

Low-Tris Buffer (LTB):

20mM Tris-HCl pH 7.9
20mM NaCl
1mM EDTA

The method used for plaque lifts was a modification of that described by Benton and Davis (1977). *E. coli* NM522 were transformed with bacteriophage M13 vectors which had been manipulated *in vitro* to contain inserts of heterologous DNA. Transformed cells were plated on minimal agar plates with the appropriate compounds for colour selection (section 2B.2.6). Colourless plaques were picked with a sterile wooden toothpick and transferred into 50 μl of LTB. A lawn of NM522 cells was prepared by adding 100 μl of a stationary phase culture in L-broth to 3.5 ml of molten top-layer agar containing 30 μl X-gal and 20 μl IPTG and plating on minimal agar plates supplemented with vitamin B1 (final concentration 0.625 μg/ml). 1 μl of M13 bacteriophage in LTB was spotted onto the cell lawn in a grid of 50 spots per plate. Bacteriophage were allowed to grow overnight and the plates were cooled to 4°C. Bacteriophage plaques were lifted onto nitrocellulose membrane disks (0.45 μm pore size; Sartorius) by carefully placing the disk on the plate, marking its position and removing it as soon as it had become moistened. It was not necessary to denature the DNA on the membranes as single stranded phage DNA was present in the plaques. Membranes were baked in a vacuum oven for 2 hours at 80°C.

2B.6.3 Transfer of DNA from agarose gels to membranes (Southern blotting)

The method used for transferring DNA from agarose gels to membranes was essentially that described by Southern (1975). DNA was fractionated by electrophoresis in an agarose gel containing ethidium bromide, and the separation of DNA fragments was

recorded by photographing the gel. The gel was soaked in approximately 5 volumes of denaturation solution for 20 minutes at room temperature with gentle shaking. The denaturation step was repeated, the gel was rinsed in H_2O and soaked in approximately 5 volumes of neutralisation buffer for 20 minutes at room temperature with gentle shaking. The neutralisation step was repeated, the gel was rinsed in H_2O and placed on top of 4 pieces of blotting paper (8" x 8" on a glass plate) saturated with 20 x SSC. Thin plastic film (Saran Wrap) was placed over the exposed areas of saturated blotting paper, in order to prevent evaporation. A nylon membrane (Nytran, Amersham International) cut to the size of the gel was soaked in 2 x SSC for 2 minutes and placed on top of the gel. Three pieces of blotting paper cut to the size of the gel were saturated with 2 x SSC and placed on top of the nylon membrane, and 20-30 pieces of dry blotting paper cut to the size of the gel were placed on top of the saturated ones. A stack of 15-20 paper hand-drying towels were placed on top of the dry blotting papers and a weight of approximately 1 kg was placed on top of the whole stack. Transfer was allowed to proceed for at least 8 hours at which time the membrane was removed from the transfer set-up and exposed to ultraviolet light from a Cromato-vue UV-transilluminator (302 nm wavelength) for 5 minutes in order to cross link DNA to the membrane.

2B.6.4 Transfer of RNA from agarose gels to membranes (Northern blotting)

RNA was fractionated by electrophoresis in a formaldehyde, agarose gel containing ethidium bromide, and the position of the ribosomal RNA bands was recorded by photographing the gel. The gel was soaked in approximately 5 volumes of 2 x SSC for 20 minutes at room temperature with gentle shaking in order to remove formaldehyde. The soaking step was repeated, the gel was rinsed in H_2O, and RNA was transferred to a nylon membrane (Nytran, Amersham International) using a set-up identical to that used for the transfer of denatured DNA from agarose gels to membranes. RNA was crosslinked to the membrane using ultraviolet light as described for single stranded DNA.

2B.6.5 Hybridisation of radio-labelled DNA probes to single stranded nucleic acids immobilised on nitrocelluose or nylon membranes

100 x Denhardt's solution (Denhardt, 1966):

2% (w/v) bovine serum albumin
2% (w/v) ficoll, molecular weight 400,000
2% (w/v) polyvinyl pyrrolidone, molecular weight 360,000

Pre-hybridisation solution:

	For 10 ml:
40% (v/v) formamide	4 ml formamide
200 μg/ml sonicated salmon sperm DNA	200 μl 10mg/ml sonicated salmon sperm DNA
6 x SSC	2.5 ml 20 x SSC
0.2% SDS	200 μl 10% (w/v) SDS
5 x Denhardt's solution	0.5 ml 100 x Denhardt's solution
	2.6 ml H_2O

Pre-hybridisation solution was prepared as follows: sonicated salmon sperm DNA was added to formamide and the solution heated for 10 minutes at 80°C to denature the DNA. The heated solution was then added to a solution of the other three components.

Hybridisation solution:

	For 10 ml:
40% formamide	4 ml formamide
200 μg/ml sonicated salmon sperm DNA	200 μl 10mg/ml sonicated salmon sperm DNA
6 x SSC	2.5 ml 20 x SSC
0.2% SDS	200 μl 10% (w/v) SDS
1 x Denhardt's solution	100 μl 100 x Denhardt's solution
	3 ml H_2O

Hybridisation solution was prepared in the same manner as pre-hybridisation solution.

A nitrocellulose or nylon membrane onto which RNA or single stranded DNA had been transfered was incubated for at least 1 hour in pre-hybridisation solution (approximately 10 ml per 100 cm^2 of membrane) in a plastic bag at 37°C with gentle shaking. The pre-hybridisation solution was then removed from the plastic bag and replaced with hybridisation solution (approximately 7 ml per 100 cm^2 of membrane). The radio-labelled DNA probe was denatured by incubating for 5 minutes at 100°C and snap-cooled by placing immediately in an ice-water bath. The probe was added to the hybridisation solution in the platic bag, the bag was resealed and incubated for at least 8 hours at 37°C with gentle shaking.

Following incubation with the radio-labelled probe, the membrane was washed in approximately 200 ml of 2 x SSC, 0.1% SDS for 15 minutes at room temperature. This wash was repeated, and the membrane was then washed in 200 ml of 0.5 x SSC, 0.1% (w/v) SDS for 1 hour at 37°C. Higher stringency could be obtained by increasing the temperature of the wash to 65°C and/or washing membranes in 0.1 x SSC, 0.1% SDS. The washed membrane was placed on blotting paper to remove excess liquid, but was not allowed to dry completely. The membrane was then sealed in a plastic bag and X-ray film (Cronex 4) was exposed to the membrane in a light-proof cassette with intensifying screens (Dupont Cronex Lightning Plus) at -70°C. Exposure times varied according to the intensity of the signal obtained.

2B.6.6 Rehybridisation

The method used to remove radio-labelled DNA probe hybridised to single stranded nucleic acid immobilised on a nylon membrane was that described in the protocols manual supplied with Gene Screen *Plus* Membranes (Dupont). 0.1 x SSC, 0.01% SDS at 100°C was poured onto the membrane which was washed in the solution for 3 minutes with gentle shaking. The solution was decanted from the membrane, and the procedure was repeated 4 times. The membrane was incubated in pre-hybridisation solution and hybridised to a new radio-labelled DNA probe as described above.

2B.7 DNA Sequencing

The method used for determination of DNA sequence was the dideoxynucleotide chain termination method based upon that described by Sanger *et al.* (1977, 1980).

2B.7.1 Preparation of single-stranded template DNA

Low-Tris buffer (LTB):

20mM Tris-HCl pH 7.9
20mM NaCl
1mM EDTA

PEG 6000 solution:

2.5M NaCl
20% (w/v) polyethylene glycol, molecular weight 6000 (Sigma)

a) Small-scale preparation:

E. coli NM522 were transformed with bacteriophage M13 vectors that had been manipulated *in vitro* to contain inserts of heterologous DNA. Transformed cells were plated on minimal agar plates with the appropriate compounds for colour selection (section 2B.2.6). Individual colourless plaques were picked with a sterile wooden toothpick and transferred into 50 μl of LTB. In order to purify single plaques, serial dilutions of LTB phage stocks were made to 10^{-5} and 10^{-6} in LTB, and 100 μl of the dilution was plated in 3.5 ml of molten top-layer agar containing 100 μl of a stationary phase culture of *E. coli* NM522 or TG1 in L-broth, 30 μl X-gal, and 20 μl IPTG on minimal plates supplemented with vitamin B1 (final concentration, 0.625 μg/ml). Bacteriophage were allowed to grow for at least 12 hours at 37°C. Individual colourless plaques were picked with a sterile toothpick and transferred into 1.5 ml of L-broth containing 15 μl of a stationary phase culture of *E. coli* NM522 or TG1 in L-broth. Cells were grown for 5.5 hours at 37°C with vigourous shaking.

Bacteriophage particles were isolated as follows: the 1.5 ml culture was transferred to a microcentrifuge tube and cells were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature. The supernatant was transferred to a fresh microcentrifuge tube and bacteriophage particles were precipitated by adding 200 μl of PEG 6000 solution. The contents of the tube were mixed by gentle inversion and incubated for 25 minutes at room temperature. The precipitated bacteriophage particles were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature. The supernatant was decanted and excess PEG 6000 solution was removed from the walls of the tube by brief centrifugation (17,000 x g) in a microcentrifuge for 1 minute at room temperature. The remaining PEG 6000 solution was removed carefully from the microcentrifuge tube by aspiration with a drawn-out, glass capillary tube. The bacteriophage pellet was resuspended in 100 μl of sequencing TE [10mM Tris-HCl pH 8.0, 0.1mM EDTA]. Bacteriophage were lysed and contaminating proteins were extracted by adding 50 μl of phenol. The liquid phases were mixed by votexing for 10 seconds, allowed to stand for 10 minutes at room temperature and mixed again by vortexing for 10 seconds. The liquid phases were separated by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature, and 85 μl of the upper, aqueous phase was recovered with care not to remove any phenol. Single stranded DNA was precipitated by adding 10 μl of 3M sodium acetate pH 5.2 and 250 μl of cold absolute ethanol (-20°C), and incubating for at least 12 hours at -20°C. Single stranded DNA was sedimented by centrifugation (17,000 x g) in a microcentrifuge for 15 minutes at 4°C, the DNA pellet was dried under vacuum and redissolved in 50 μl of sequencing TE.

b) Large-scale preparation

Single plaques from a recombinant bacteriophage M13 stock in LTB were isolated as described for small-scale preparation. As for small-scale preparation, individual plaques were picked with a sterile toothpick and transferred to 1.5 ml of L-broth containing 15 μl of a stationary phase culture of *E. coli* TG1 in L-broth. Cells were grown for 5.5 hours at 37°C with vigourous shaking, the 1.5 ml culture was transferred to a microcentrifuge tube, and cells were sedimented by centrifugation. The supernatant was transferred to a fresh microcentrifuge tube. 100 ml of L-broth was inoculated with 1 ml of a stationary phase culture of TG1 cells in L-broth, and the cells were grown to OD_{650nm} 0.3 at 37°C with shaking. The supernatant from the 1.5 ml bacteriophage culture was used to

innoculate the 100 ml culture of TG1 cells, and the culture was grown for 4 hours at 37°C with shaking. Cells were sedimented from suspension by centrufugation (12,000 x g) for 30 minutes at 4°C, and the supernatant was transferred to fresh centrifuge tubes. Bacteriophage particles were precipitated by adding 0.2 volumes of PEG 6000 solution, the contents of the tubes were mixed by gentle inversion and incubated for 25 minutes at room temperature. The precipitated bacteriophage particles were sedimented by centrifugation (12,000 x g) for 5 minutes at room temperature. The supernatant was decanted, and excess PEG 6000 solution was removed from the walls of the centrifuge tube by brief centrifugation (12,000 x g) for 1 minute at room temperature. The remaining PEG 6000 solution was removed carefully from the centrifuge tube by aspiration with a drawn-out, glass Pasteur pipette. The bacteriophage pellet was resuspended in 0.5 ml of sequencing TE [10mM Tris-HCl pH 8.0, 0.1mM EDTA] and transferred to a microcentrifuge tube. Bacteriophage particles were re-precipitated by adding 200 μl of PEG 6000 solution, the contents of the tube were mixed by gentle inversion and incubated for 15 minutes at room temperature. The precipitated bacteriophage particles were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature. The supernatant was decanted, and excess PEG 6000 solution was removed from the walls of the tube by brief centrifugation (17,000 X g) in a microcentrifuge for 1 minute at room temperature. The remaining PEG 6000 solution was removed from the microcentrifuge tube by aspiration with a drawn-out, glass capillary tube. The bacteriophage pellet was resuspended in 0.5 ml of sequencing TE; bacteriophage were lysed and contaminating proteins were extracted by adding 200 μl of phenol. The liquid phases were mixed by vortexing for 20 seconds, allowed to stand for 15 minutes at room temperature and mixed again by vortexing for 20 seconds. The phases were separated by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes at room temperature, and the upper, aqueous phase was recovered. Contaminating proteins were removed by repeated extraction with 200 μl of phenol. The upper, aqueous layer was recovered and remaining traces of phenol were extracted by adding 0.5 ml of diethyl ether. The lower, aqueous phase was recovered and re-extracted with 0.5 ml of diethyl ether. The lower, aqueous phase was recovered and extracted with 0.5 ml of chloroform. The upper, aqueous phase was recovered, and single stranded bacteriophage DNA was precipitated with ethanol. The DNA pellet was dried under vacuum and redissolved in 100 μl of sequencing TE.

2B.7.2 Dideoxynucleotide chain-termination sequencing reactions

10 x TM:

100mM Tris-HCl pH 8.5

50mM $MgCl_2$

Oligodeoxynucleotide sequencing primers:

5´d(GTAAAACGACGGCCAGT)3´	Bacteriophage M13 universal sequencing primer (20 nucleotides from the polylinker site); 17 bases.
5´d(CGCCAACTTACAAGGCC)3´	HBV sense strand, nucleotide positions 2382-2398; 17 bases.
5´d(ATGGCTGCTAGGCTGTG)3´	HBV sense strand, nucleotide positions 2656-2672; 17 bases.
5´d(GCACGTCGCATGGAGA)3´	HBV sense-strand, nucleotide positions 2881-2896; 16 bases.
5´d(GCGGCTAGGAGTTCCGC)3´	HBV anti-sense strand, nucleotide positions 2571-2555; 17 bases.

Oligodeoxynucleotide sequencing primers were synthesised by the OSWEL DNA service, Department of Chemistry, University of Edinburgh, using an Applied Biosystems Inc, 380B, DNA Synthesizer and were diluted in H_2O to a concentration of 1 pmole/μl.

Primer mixes:

a) Primer mix for complete sequencing (4 chain termination reactions):

1.5 μl oligodeoxynucleotide primer (1 pmole/μl)

1.5 μl 10 x TM

10 μl single stranded template DNA (100 ng/μl)

b) Primer mix for single dideoxynucleotide tracking (per clone):

0.4 μl oligodeoxynucleotide primer (1 pmole/μl)
0.4 μl 10 x TM
2.4 μl H_2O
3 μl single stranded template DNA (100 ng/μl)

Chain-termination mixes:

	Chain-Termination Mix (numbers are in μl)			
Nucleotide	T°	C°	G°	A°
0.5mM dTTP	10	200	200	200
0.5mM dCTP	200	10	200	200
0.5mM dGTP	200	200	10	200
10mM ddTTP	20			
10mM ddCTP		3.2		
10mM ddGTP			6.4	
10mM ddATP				0.5
sequencing TE	400	400	400	200

Klenow reaction mix (per template; 4 chain termination reactions):

4 μCi [α-^{35}S]dATP (8 μCi/μl; Amersham)
1.5 units *E. coli* DNA polymerase I (Klenow fragment) (5 U/μl; Boehringer)
0.8 μl 10mM DTT
6.5 μl 10mM Tris-HCl pH 8.5

dNTP chase mix:

dTTP, dCTP, dGTP, dATP each at 0.25mM

Formamide dye:

0.1% (w/v) xylene cyanol
0.1% (w/v) bromophenol blue
10mM EDTA
in formamide

Single stranded bacteriophage M13 DNA containing an insert of heterologous DNA was primed for DNA polymerisation by annealing to a synthetic oligodeoxynucleotide containing sequence complementary to the *lac*Z gene of the M13 vector or to the DNA insert in the M13 vector. 0.5 pmole of primer DNA was mixed with 1 μg of template DNA in 1 x TM, the solution was incubated for 5 minutes at 80°C and slowly cooled to room temperature over 20 minutes. Sequencing reactions were carried out in microtitre plates. 2 μl of template DNA with annealed primer was dispensed into each of 4 wells followed by 2 μl of one of the chain-termination mixes (G°, A°, T°, or C°), and the solutions were mixed by tapping the plate. 2 μl of Klenow reaction mix was added to each well, the solutions were mixed by tapping the plate and incubated for 25 minutes at room temperature. 2 μl of dNTP chase mix was added to each well, the solutions were mixed by tapping the plate and incubated for a further 20 minutes at room temperature. For single-track sequencing the reaction was carried out for only one of the chain-termination mixes per template and the rest of the reactions were performed as described above. Sequencing reactions were stopped by adding 2 μl of formamide dye to each well and double stranded reaction products were denatured by incubating the solutions for 15 minutes at 80°C.

2B.7.3 Resolution of sequencing reaction products by electrophoresis in urea-polyacrylamide gels

Urea-polyacrylamide solution:

17 g urea
6 ml 40% (w/v) acrylamide [38% (w/v) acrylamide, 2% (w/v) *N,N'*-methylene bisacrylamide
4 ml 10 x TBE
17 ml H_2O

DNA sequencing reaction products were resolved in 6% acrylamide, 7M urea gels on a vertical gel apparatus. 240 μl of 10% (w/v) ammonium persulphate and 35 μl of *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED) were added to the urea-polyacrylamide solution, the solution was poured between two glass plates with gel dimensions 380mm x 200mm x 0.5 mm, and allowed to polymerise with the well comb in place.

The heated sequencing reactions were snap-cooled on ice and approximately 2 μl of each sample was loaded onto the gel using a drawn-out, glass capillary tube. The DNA sequencing products were separated by electrophoresis using 1 x TBE running buffer at constant power of 25-30 watts (ca. 20-30 mA, 1.1-1.5 kV). Electrophoresis was continued until the required fragment separation was achieved (bromophenol blue migrates with DNA fragments of about 25 nucleotides in length, xylene cyanol migrates with DNA fragments of about 75 nucleotides in length). After electrophoresis the gel was fixed by immersion in 10% (v/v) acetic acid, 10% (v/v) methanol for 15 minutes at room temperature. The gel was transferred to blotting paper, covered in thin plastic film (Saran Wrap) and dried on a gel slab drier. X-ray film was exposed to the dried gel for 16 hours or longer in a light-proof cassette at -70°C to prevent the gel from sticking to the film.

2B.8 Amplification of mRNA Using the Polymerase Chain Reaction (PCR)

10 x Activity Grade™ Buffer (IBI):

100mM Tris-HCl pH 8.3
0.5M KCl
15mM $MgCl_2$
0.1% (w/v) gelatin
0.1% (v/v) Tween 20
0.1% (v/v) NP-40

Oligodeoxynucleotide primers:

5´d(GGCTGCAGGGGTCACCATATTCTTGGGAACAA)3´

HBV sense strand, nucleotide positions 914-937 with eight additional nucleotides at the 5´ end including a cleavage site for restriction endoculease *Pst*I. T_m = 70°C.

5´d(GGGAATTCGCGGGGTAGGCTGCCGTCCT)3´

HBV anti-sense strand, nucleotide positions 1236-1217 with eight additional nucleotides at the 5´ end including a cleavage site for restriction endonuclease *Eco*RI. T_m = 70°C.

5´d(GCTATTCCAGAAGTAGTGAGGAG)3´

SV40 nucleotide positions 5228-5206. T_m = 68°C.

5´d(GCCCCAAAGCCACCCAAGGC)3´

HBV anti-sense strand, nucleotide positions 3182-3163. T_m = 68°C.

Oligodeoxynucleotides were synthesised by the OSWEL DNA service, Department of Chemistry, University of Edinburgh, using an Applied Biosystems Inc., 380B, DNA Synthesizer and were diluted in H_2O to a concentration of 10 pmoles/μl.

The reverse transcriptase activity of *Thermus aquaticus* DNA polymerase (Jones and Foulkes, 1989; Tse and Forget 1990) was exploited to amplify sequnces directly from RNA. RNA was prepared from monolayers of mammalian cells in culture and stored in aliquots of approximately 40 μg at -70°C as an ethanol precipitate as described above (section 2B.2.3). RNA was sedimented by centrifugation (17,000 x g) in a microcentrifuge for 15 minutes at 4°C, and the superanatant was removed carefully by aspiration with a drawn-out, glass capillary tube. The RNA pellet was redissolved in 45 μl H_2O and 5 μl of 10 x DNaseI buffer [1M sodium acetate, 100 mM $MgCl_2$] was added. 1 μl of DNaseI (23 U/μl, RNase free; Boehringer) was added to the RNA solution, and it was incubated for 30 minutes at 37°C. The DNase treated solution was split into two, 25 μl parts. 1 μl of RNase A (10 mg/ml; Sigma) was added to one of the parts, and it was incubated for a further 30 minutes at 37°C. Contaminating proteins in both parts were extracted with

PCI, and nucleic acid was precipitated with ethanol. The supernatants were carefully removed by aspiration with a drawn-out, glass capillary tube and the nucleic acid pellets were redissolved in 50 μl H_2O. 1 μl RNase inhibitor (40 U/μl RNAsin; Promega) was added to each sample. PCR reactions were carried out in the following 50 μl reaction mixture:

5 μl RNA sample (approx. 2 μg in total of DNase treated material) or RNase treated sample
5 μl 10 x Activity Grade^{TM} Buffer (IBI)
1 μl 10mM each dGTP, dATP, dTTP, dCTP
1 μl sense strand primer (10 pmoles/μl)
1 μl anti-sense strand primer (10 pmoles/μl)
0.5 μl *Thermus aquaticus* DNA polymerase (4 U/μl; IBI)
36.5 μl H_2O
overlayed with 50 μl sterile paraffin oil

PCR reactions were carried out using a Techne PHC-2 Dri-Block with 30 cycles of the following reaction times:

denaturation: 93°C, 1.5 minutes
annealing: 59°C, 1.5 minutes
polymerisation: 70°C, 1 minute.

The products of PCR reactions were visualised by "Southern" hybridisation. The DNA products in 10 μl of PCR reaction mixture were fractionated by electrophoresis in an agarose gel and transferred to a nylon membrane (Nytran, Amersham Interantional). DNA fragments were detected by hybridisation to a radio-labelled DNA probe representing sequences internal to both oligodeoxynucleotide primers.

2B.9 Site Directed Mutagenesis (SDM)

The method used for SDM was developed by D. Melton (personal communication).

10 x polynucleotide kinase buffer:

0.5M Tris-HCl pH 7.5
100mM $MgCl_2$
100mM DTT
10mM ATP
0.5 mg/ml BSA

10 x TM:

100mM Tris-HCl pH 8.5
50mM $MgCl_2$

Sequencing TE:

10mM Tris-HCl pH 8.0
0.1mM EDTA

dNTP chase mix:

dTTP, dCTP, dGTP, dATP each at 0.25mM

Mutant oligodeoxynucleotide primer:

5´d(CGGCCAGGTCTGTGCCCTGGCTGGGGC)3´

HBV sense strand, nucleotide positions 2444-2459/2485-2495 for deletion of nucleotides 2460-2484.

Oligodeoxynucleotide was synthesised by the OSWEL DNA Service, Department of Chemistry, University of Edinburgh, using an Applied Biosystems Inc., 380B, DNA synthesiser. Oligodeoxynucleotide was supplied at a concentration of 40 pmoles/μl in H_2O and used undiluted.

2B.9.1 Phosphorylation of 5´ end of oligodeoxynucleotide with T4 polynucleotide kinase

T4 polynucleotide kinase was prepared by S. Bruce as described by Midgley and Murray (1985). Phosphorylation of the 5´ end of the mutant oligodeoxynucleotide was carried out for 1.5 hours at 37°C in the following reaction mixture:

6 μl oligodeoxynucleotide (40 pmoles/μl)
1 μl 10 x polynucleotide kinase buffer
2 μl T4 polynucleotide kinase (0.5 U/μl)
1 μl H_2O.

40 μl H_2O were added after the reaction time, contaminating proteins were extracted with PCI and nucleic acid was precipitated with ethanol. The nucleic acid pellet was dried under vacuum and redissolved in 12 μl H_2O.

2B.9.2 Synthesis of mutant strand DNA and recovery of mutant clones

The bacteriophage M13 single stranded DNA template used for mutagenesis was the vector M13mp19 with 2.3 kb of HBV DNA (nucleotide positions 1004-3182/1-82) inserted at the polylinker site. The phosphorylated, mutant primer and the 17-base universal primer for bacteriophage M13 (section 2B.7.2) were annealed to the template DNA by incubating the following mixture for 5 minutes at 80°C and cooling slowly over 20 minutes to room temperature:

12 μl phosporylated mutant primer (20 pmoles/μl)
3 μl universal primer (1 pmole/μl)
3 μl single stranded template DNA (1 μg/μl)
2 μl 10 x TM

DNA polymerisation was carried out for 1.5 hours at room temperature in the following reaction mixture:

20 μl single stranded template DNA with primers annealed
20 μl dNTP chase mix
1 μl 50mM ATP
1.5 μl *E. coli* DNA polymerase I (Klenow fragment) (5 U/μl; Boehringer)
5 μl T4 DNA ligase (1 U/μl; Boehringer)
2.5 μl sequencing TE

After the reaction time, contaminating proteins were extracted with PCI and remaining traces of PCI were extracted with an equal volume of butan-1-ol. The lower, aqueous phase was recovered, and DNA was precipitated with ethanol. The DNA pellet was dried under vacuum and redissolved in 32 μl H_2O. The HBV DNA insert was now double stranded, and it was excised from the bacteriophage M13 vector DNA by digestion with *Eco*RI and *Hind*III. The cleavage products were fractionated by electrophoresis in a low-melting-temperature agarose gel and the 2.3 kb HBV insert DNA fragment was purified as described above (section 2B.4.2). This DNA fragment was ligated to M13mp19 vector DNA that had been linearised by digestion with *Eco*RI and *Hind*III. *E. coli* BMH71-18 *mut*L cells were transformed with ligation mixture and recombinant plaques were isolated. M13 clones containing a mutated HBV DNA insert were identified by T-track sequencing. 1 ng of mutant template DNA was used to transform *E. coli* strain TG1. A single, colourless plaque was isolated for small-scale preparation of RF DNA as described above (section 2B.2.4c). The insert of HBV DNA was excised from the bacteriophage M13 vector DNA by digestion with *Eco*RI and *Hind*III, and the resulting 2.3 kb HBV DNA fragment was ligated to pUC8 linearised by digestion with *Eco*RI and *Hind*III.

2B.10 Techniques for Analysis of Proteins.

2B.10.1 Radio-immunoassay for detection of HBsAg

The medium was recovered from confluent monolayers of hepatoma cells 3.5-4.5 days post-transfection with HBsAg expression vectors. In some cases HBsAg was concentrated from the culture medium by ultracentrifugation (150,000 x g for 5 to 16 hours) and resuspended in 1/20th or 1/30th volume of PBS for assay. Secreted HBsAg was detected by radio-immunoassay using the AUSRIA II-125 kit (Abbott Laboratories). The method used was procedure A or **B** as recommended by the manufacturer. 200 μl of sample was added to each well containing a polystyrene bead coated with guinea pig antibody to HBsAg and was incubated for 2 hours at 45°C or for 16 hours at room temperature. The sample was removed by aspiration and the beads were washed 10 times by filling the well each time with 1 ml H_2O and removing it by aspiration. 200 μl of [^{125}I]-anti-HBsAg (human) (0.74 μCi/ml) was added to each well and incubated with the beads for 1 hour at 45°C. The liquid was removed by aspiration and the beads were washed as described above. The beads were transferred to counting phials and counted in an LKB Mini-Gamma counter.

For detection of HBsAg particles containing middle-S (preS2/surface) polypeptides, polystyrene beads were incubated at room temperature for at least 4 hours in a solution of 200mM $NaHCO_3$ pH 9.2, containing 60 μg/ml anti-preS2 monoclonal antibody, Q19/10 (Heerman *et al.*, 1988). Beads were dried on absorbent paper and used in the radio-immunoassay described above in place of the beads coated with guinea pig anti-HBsAg. For detection of HBsAg particles containing large-S (preS1/preS2/surface) polypeptides, beads were coated with anti-preS1 monoclonal antibody, MA18/7 (Heerman *et al.*, 1984), and used in the radio-immunoassay as described above.

2B.10.2 Lowry protein assay

Solutions:

Lowry reagent:

2% (w/v) Na_2CO_3
0.01% (w/v) $CuSO_4$
0.02% (w/v) tri-sodium citrate
100mM NaOH

The method used to determine the concentration of protein in solution was that decribed by Lowry *et al.* (1951). Protein sample in 0.4 ml was added to 2ml of Lowry reagent; the solution was mixed by inversion and incubated for 10 minutes at room temperature. 200 μl of 50% (v/v) Folin-Ciocaltea's phenol reagent (Sigma) was added, mixed by inversion, and the mixture was incubated for 30 minutes at room temperature. OD_{650nm} was recorded for the sample; the assay was calibrated using BSA at a concentration of 1 to 400 μg/ml.

2B.10.3 Electrophoresis of proteins in polyacrylamide gels

Resolving gel solutions (50 ml):

7% acrylamide		12.5% acrylamide
11.6 ml	30% (w/v) acrylamide	20.8 ml
11.6 ml	0.2% (w/v) *N,N'*-methylene bisacrylamide	20.8 ml
6.25 ml	3M Tris-HCl pH 8.8	6.25 ml
20.5 ml	H_2O	2.2 ml

Stacking gel solution (15ml, 3.75% acrylamide):

1.88 ml 30% (w/v) acrylamide
1.88 ml 0.2% (w/v) *N,N´*-methylene bisacrylamide
1.88 ml 1M Tris-HCl pH 6.8
9.36 ml H_2O

2 x PAGE loading buffer:

120mM Tris-HCl pH 6.8
4% (w/v) SDS
20% (v/v) glycerol
60 mM DTT (added to immediately prior to use)

10 x PAGE running buffer

0.25M Tris-HCl
2M glycine
1% (w/v) SDS

Proteins were separated on the basis of their molecular weight by polyacrylamide gel electrophoresis (PAGE). The method used was the discontinuous buffer system described by Laemmli (1970). 10 ml of resolving gel solution was placed in a separate beaker and 150 μl of 10% (w/v) ammonium persulphate and 10 μl of TEMED were added. The solution was poured into a mould into which the bottom of the glass plates for PAGE had been placed and was allowed to polymerise for 15 minutes to seal the gap between the bottoms of the plates. 0.4 ml of 10% (w/v) SDS, 200 μl of 10% (w/v) ammonium persulphate and 20 μl of TEMED were added to the remaining 40 ml of resolving gel solution, and the solution was poured between the sealed glass plates with gel demensions 170mm x 150mm x 1.5mm. The solution between the glass plates was overlaid with H_2O and left to polymerise for at least 1 hour. 150 μl of 10% (w/v) SDS, 100 μl of 10% (w/v) ammonium persulphate and 10 μl of TEMED were added to the stacking gel solution, the water was removed from above the polymerised resolving gel, and the stacking gel was poured on top of the resolving gel with gel dimensions 50mm x 150mm x 1.5mm. The

stacking gel was allowed to polymerise with the gel comb in place for at least 30 minutes.

An equal volume of 2 x PAGE loading buffer was added to protein samples and they were incubated for 5 minutes at 100°C before loading onto the gel. Electrophoresis was carried out on a vertical apparatus using 1 x PAGE running buffer for 5-6 hours at 25 mA or for 12-16 hours at 6 mA until the desired separation was achieved. Low molecular weight markers (Pharmacia) were: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anydrase (30 kD), trypsin inhibitor (20.1 kD) and α-lactalbumin (14.4 kD). High molecular with markers (Sigma) were: myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), bovine albumin (66 kD), egg albumin (45 kD) and carbonic anhydrase (29 kD).

2B.10.4 Staining of proteins separated in polyacrylamide gels

After electrophoresis, polyacrylamide gels were removed from between glass plates and incubated with shaking for 1 hour at room temperature in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid containing 0.2% (w/v) Coomassie brilliant blue R (Sigma). Gels were destained in 40% (v/v) methanol, 10% (v/v) acetic acid. For storage, gels were dried onto blotting paper using a slab gel drier.

2B.10.5 Transfer of proteins separated in polyacrylamide gels to membranes (Western blotting)

Transfer buffer:

25mM Tris-HCl
0.2M glycine
20% (v/v) methanol

The method used for transfer of proteins separated on polyacrylamide gels to membranes was essentially that described by Towbin *et al.* (1979). After electrophoresis, polyacrylamide gels were removed from between glass plates and a cassette was constructed containing the following components saturated with transfer buffer and layered in the following order: Scotchbrite pad, 3 sheets of blotting paper cut to the size

of the gel, nitrocellulose membrane (0.45 μm pore size; Schleicher and Schull) cut to the size of the gel, 3 sheets of blotting paper cut to the size of the gel, Scotchbrite pad. The cassette was closed and placed with the nitrocellulose membrane between the gel and the anode in a Bio-Rad electrotransfer apparatus filled with transfer buffer. Proteins were transfered at 75 V for 4 hours or 40 V for 16 hours.

Proteins transferred to the nitrocellose membrane could be stained reversibly by incubation of the membrane in a solution of 3% (w/v) trichloro-acetic acid and 0.5% (w/v) Ponceau S (Sigma) for 30 seconds at room temperature and destaining with H_2O. The stain was removed by incubation of the membrane in protein blocking solution (TS/Milk, section 2B.10.8). Proteins transferred to the nitrocellose membrane could be stained permanently by incubation of the membrane in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid containing 0.1% (w/v) amido black NB (Sigma) for 30 seconds at room temperature. The membrane was destained by washing in several changes of 40% (v/v) methanol, 10% (v/v) acetic acid.

2B.10.6 Spotting of proteins onto nitrocellulose membranes

50 μl of protein preparation was spotted onto a nitrocellulose membrane using a vacuum apparatus (Hybri.Dot^{TM} Manifold; Bethesda Research Laboratories)

2B.10.7 Purification of rabbit antiserum

Antibodies in rabbit 98 anti-HBxAg serum and rabbit 87 anti-HBcAg IgG preparation that cross-reacted with endogenous *E. coli* RB791 proteins were removed by preadsorbing the preparations against a lysate of these cells. 10 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with a single colony of *E. coli* RB791, and cells were grown to stationary phase at 37°C with shaking. The cells were sedimented from suspension by centrifugation (12,000 x g) for 10 minutes at 4°C, and the cell pellet was resuspended in 1 ml of TS [10mM Tris-HCl pH 7.4, 150mM NaCl]. 100 μl of 10% (w/v) SDS was added, and the suspension was incubated for 5 minutes at 100°C. This lysate was diluted 10 fold in TS. Equal volumes of *E. coli* cell lysate and either Rabbit 87 IgG preparation or Rabbit 98 antiserum were mixed by rotation for 12-16 hours at 4°C. Antibody-antigen complexes were removed by centrifugation (17,000 x g) in a microcentrifuge for 15

minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and an equal volume of cell lysate was added again. The procedure was repeated 4 times and the pre-adsorbed antiserum was stored at -20°C.

Antibodies in rabbit 98 anti-HBxAg serum that cross-reacted with the λcro/β-galactosidase fusion protein encoded by the pEX vector series, and antibodies that cross-reacted with endogenous *E. coli* NF1 proteins were removed by pre-adsorbing the antiserum against a lysate from *E. coli* NF1 cells producing the pEX vector fusion protein. 5 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with a single colony of *E. coli* NF1 harbouring plasmid vector pEX2. The cells were grown to stationary phase at 30°C with shaking. 10 ml of L-broth containing 100 μg/ml ampicillin was inoculated with 100 μl of the stationary phase culture, and cells were grown to OD_{650nm} 0.5 at 30°C with shaking. Expression of the fusion protein from the λPr promoter contained in the pEX vector was induced by incubation of the culture for 30 minutes at 42°C with shaking. The culture was then returned to 30°C for a further 30 minutes with shaking. Cells were sedimented from the induced culture by centrifugation (12,000 x g) for 10 minutes at 4°C, and the cell pellet was resuspended in 1 ml of TS [10mM Tris-HCl pH 7.4, 150mM NaCl]. The cell lysate was prepared as described above for *E. coli* RB791 and the antiserum was preadsorbed as described above.

2B.10.8 Immunological detection of proteins immobilised on nitrocellulose filters

10 x Tris-HCl/NaCl (TS):

100mM Tris-HCl pH 8.1
150mM NaCl
0.5% Tween 20

TS/Milk:

5% (w/v) non-fat milk powder (Marvel) in 1 x TS
0.1% (w/v) sodium azide

Developing solution:

100mM Tris-HCl pH 9.5
100mM NaCl
50mM $MgCl_2$
0.33 mg/ml nitro-blue-tetrazolium (NBT)
0.25 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)
(NBT and BCIP added immediately prior to use)

Protein samples were electroblotted or spotted onto a nitrocellulose membrane, and the membrane was incubated for at least 1 hour at room temperature with shaking in TS/Milk in order to block non-specific binding of protein probes to the membrane. The blocking solution was removed, and the membrane was incubated with the appropriate dilution if primary antibody in TS/Milk (approximately 10 ml per 100 cm^2 of membrane) for 4-16 hours at room temperature with gentle shaking. The primary antibody solution was removed, and the membrane was washed in 5 changes of 1 x TS over a period of 30 minutes. Depending on the animal source of the primary antibody, the secondary antibody was either goat anti-rabbit IgG (F_c) alkaline phosphatase conjugate or goat anti-human IgG (F_c) alkaline phosphatase conjugate (1 mg/ml; Promega Biotec). The washed membrane was incubated with a 1:7500 dilution of secondary antibody in TS/Milk for 2 hours at room temperature with gentle shaking. The membrane was washed in 1 x TS as before, and the presence of bound alkaline phosphatatase was detected by incubation of the membrane in developing solution for 2-15 minutes at room temperature. The reaction was stopped by washing the memebrane in several changes of H_2O.

2B.10.9 Preparation of β-galactosidase fusion proteins from *E. coli*

The method used to prepare β-galactosidase fusion proteins was essentially that described by Stanley and Luzio (1984). Portions of the HBxAg coding sequence were fused in-frame to the 3´ end of the coding sequence for β-galactosidase contained in the plasmid pEX2 or pEX3. A single colony of *E. coli* NF1 harbouring one of the pEX plasmid derivatives was used to innoculate 5 ml of L-broth containing ampicillin (100 μg/ml), and cells were grown to stationary phase at 30°C with shaking. 1.5 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with 30 μl of stationary phase culture and cells were

grown to OD_{650nm} 0.5 at 30°C with shaking. Expression of the fusion protein from the λPr contained in the pEX vector was induced by incubation of the culture for 30 minutes at 42°C with shaking. The culture was then returned to 30°C for a further 30 minutes with shaking. 1 ml of the induced cell culture was transferred to a microcentrifuge tube, and cells were sedimented from suspension by centrifugation (17,000 x g) in a microcentrifuge for 1 minute at room temperature. The cell pellet was resuspended in 40 μl PAGE loading buffer, cells were lysed and proteins were denatured by incubation at 100°C for 5 minutes, and samples were loaded onto a 7% polyacrylamide gel.

2B.10.10 Purification of HBcX produced in *E. coli*

HBcX is a fusion protein of HBcAg and HBxAg encoded by plasmid pHBcX whose construction is described in section 5.2.1. The methods used to purify HBcX were modifications of those described by Stahl and Murray (1989).

Lysis Buffer:

50mM Tris-HCl pH 8.0
1% (v/v) Triton-X100
0.1mM phenylmethylsulphonyl fluoride (PMSF)

Column matrices, sizes and equilibration buffers are listed in table 2.1. All column matrices were from Pharmacia. Columns were equilibrated with 400 ml of buffer at a flow rate of 20 ml per hour. Proteins were eluted from the columns in the amount of buffer indicated in the text at a flow rate of 10 ml per hour. 3.3 ml fractions of eluate were collected and assayed for the presence of HBcX by, a) immunodetection with rabbit 98 anti-HBxAg serum or rabbit 87 anti-HBcAg IgG preparation of proteins spotted onto nitrocellulose membrane, b) immunodetection with rabbit 98 anti-HBxAg serum of proteins fractionated by PAGE in 12.5% acrylamide gel and electroblotted to nitrocellulose membrane, or c) staining of proteins fractionated by PAGE with Coomassie blue. Other methods employed for detection of HBcX are indicated in the text.

Table 2.1 Columns used in purification of HBcX

Matrix	Size	Equilibration buffer
Sepharose CL-4B	2.5 cm diameter X 11 cm	100mM $NaHCO_3$ pH 7.0
Sephacryl S-200	2.5 cm diameter X 12.5 cm	100mM $NaHCO_3$ pH 9.6
Sephacryl S-200	2.5 cm diameter X 12.5 cm	10mM Tris-HCl pH 7.0
Sephacryl S-200	2.5 cm diameter X 10.5 cm	8M Urea in 10mM Tris-HCl pH7.0
carboxymethyl (CM) - Sepharose	1.5 cm diameter X 11 cm	10mM Tris-HCl pH 7.0
DEAE-Sepharose	1.5 cm diameter X 11.5 cm	10mM Tris-HCl pH 7.0

a) Small-scale preparation

10 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with a single colony of *E. coli* RB691 harbouring plasmid pHBcX, and cells were grown to stationary phase at 37°C with shaking. 500 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with 10 ml of stationary phase culture, cells were grown to OD_{650nm} 1.0 at 37°C with shaking, and 2.5 ml of 100mM IPTG in dimethylformamide was added (final concentration, 0.5mM). The cells were grown for 16 hours at 37°C with shaking and were sedimented from suspension by centrifugation (17,000 x g) for 10 minutes at 4°C. The cell pellet was resuspended in 100 ml of L-broth, and cells were sedimented from suspension again by centrifugation (17,000 x g) for 10 minutes at 4°C. The cell pellet was resuspended in 10 ml of lysis buffer, the suspension was transferred to a plastic centrifuge tube and sonicated for 8 x 30 seconds (Soniprobe, Dawe Instruments type 7530A) to lyse the cells. The cellular debris in the lysate was sedimented by centrifugation (12,000 x g) for 10 minutes at 4°C, and the supernatant (designated "crude extract") was recovered.

4 ml of crude extract was layered onto the column of Sepharose CL-4B, and protein was eluted in 200 ml of 100mM $NaHCO_3$ pH 7.0. Fractions containing HBcX were combined and fusion protein was sedimented by ultracentrifugation (160,000 x g) for 1.5 hours at 4°C. The protein pellet was resuspended in 0.5 ml of 100mM $NaHCO_3$ pH 7.0 containing 0.1mM PMSF and stored at -20°C.

b) Large-scale preparation 1.

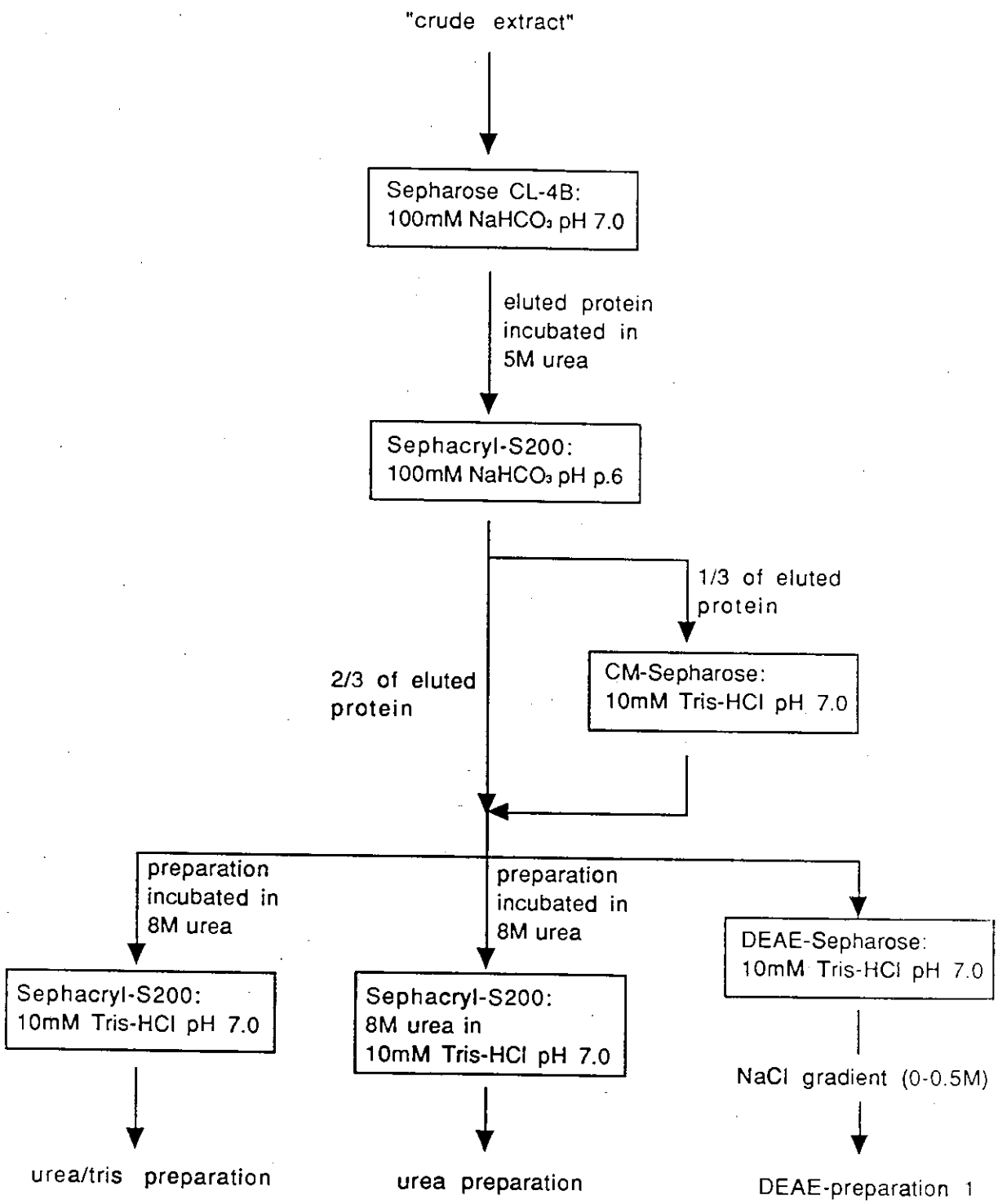
Purification steps used in this preparation are diagrammed in figure 2.1.

200 ml of L-broth containing ampicillin (100 μg/ml) was inoculated with a single colony of *E. coli* RB791 harbouring plasmid pHBcX, and cells were grown to stationary phase at 37°C with shaking. 5 x 2 litres of L-broth containing ampicillin (100 μg/ml) was inoculated with 40 ml/2l of stationary phase culture, cells were grown to OD_{650nm} 1.0 at 37°C with shaking, and 1ml/2l of 1M IPTG in dimthylformamide was added (final concentration, 0.5mM). The cells were grown for 16 hours at 37°C with shaking and were sedimented from suspension by contiuous-flow centrifugation. The cell paste was resuspended in 200ml of lysis buffer, and cells were broken by passing through a French press (5 x 15 seconds at 8000 psi). The cellular debris in the lysate was sedimented by centrifugation (12,000 x g) for 20 minutes at 4°C, and the supernatant was recovered. Fusion protein was sedimented from crude extract by ultracentrifugation (160,000 x g) for 1.5 hours at 4°C, and the protein pellets were resuspended in a total of 6ml of 100mM $NaHCO_3$ pH 7.0 containing 0.1mM PMSF. This preparation is designated "crude extract" in figures 2.1 and 2.2.

3 ml of crude extract was layered onto the column of Sepharose CL-4B, and protein was eluted in 100 ml of 100mM $NaHCO_3$ pH 7.0. Fractions containing HBcX were combined, and fusion protein was sedimented as above. The protein pellet was resuspended in 1.5 ml of 5M urea in 100mM $NaHCO_3$ pH 9.6. This solution was heated to 37°C for 1 hour, and layered onto the column of Sephacryl S-200 equilibrated in 100mM $NaHCO_3$ pH 9.6. Protein was eluted in 200ml of this buffer; fractions containing HBcX were combined, and fusion protein was sedimented as above. The protein pellet was resuspended in 1.5 ml of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF. One third of the HBcX preparation (0.5 ml) was layered onto the column of CM-Sepharose, and protein was eluted in 200 ml of 10mM Tris-HCl pH 7.0. Fusion protein was located in a single fraction by Lowry protein assay and sedimented as above. The protein pellet was resuspended in 100 μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF, and the purity of the preparation was assayed by PAGE in 12.5% acrylamide gel. The 100 μl preparation was recombined with the 1 ml of preparation remaining after gel-filtration in Sephacryl-S200.

FIGURE 2.1 Scheme for purification of HBcX. Large-scale preparation 1.

Column chromatography stages of purification are indicated in boxes along with the buffer used for equilibration of each column. Details of the purification procedure are provided in the text (section 2B.10.10b).


"crude extract"
Sepharose CL-4B:
100mM NaHCO3 pH 7.0
eluted protein
incubated in
5M urea
Sephacryl-S200:
100mM NaHCO3 pH p.6
1/3 of eluted
protein
2/3 of eluted
protein
CM-Sepharose:
10mM Tris-HCl pH 7.0
preparation
incubated in
8M urea
preparation
incubated in
8M urea
DEAE-Sepharose:
10mM Tris-HCl pH 7.0
Sephacryl-S200:
10mM Tris-HCl pH 7.0
Sephacryl-S200:
8M urea in
10mM Tris-HCl pH 7.0
NaCl gradient (0-0.5M)
urea/tris preparation
urea preparation
DEAE-preparation 1

The protein preparation was split into three parts of 0.35 ml. 1.7 ml of 8M urea in 10mM Tris-HCl pH 7.0 was added to one of the parts, the solution was heated to 37°C for 1 hour, and layered onto the column of Sephacryl S-200 equilibrated in 10mM Tris-HCl pH 7.0. Protein was eluted in 200 ml of 10mM Tris-HCl pH 7.0. Fusion protein was sedimented as above from each HBcX-positive fraction separately, and each protein pellet was resuspended in 100 μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

1.7 ml of 8M urea in 10mM Tris-HCl pH 7.0 was added to the second part of the HBcX preparation, the solution was heated to 37°C for one hour and layered onto the column of Sephacryl S-200 equilibrated in the 8M urea solution. Protein was eluted in 200 ml of this solution, and fusion protein was sedimented as above from each HBcX-positive fraction separately. Each protein pellet was resuspended in 100 μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

The third, part of the HBcX preparation was layered onto the column of DEAE-Sepharose, and was bound to the column by washing in 20 ml of 10mM Tris-HCl pH 7.0 at a flow rate of 10 ml/hour. Protein was eluted from the column with a 100 ml continuous gradient of NaCl (0 - 0.5M) in 10mM Tris-HCl pH 7.0. Fractions containing HBcX were combined, and fusion protein was sedimented as above. The protein pellet was resuspended in 200 μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

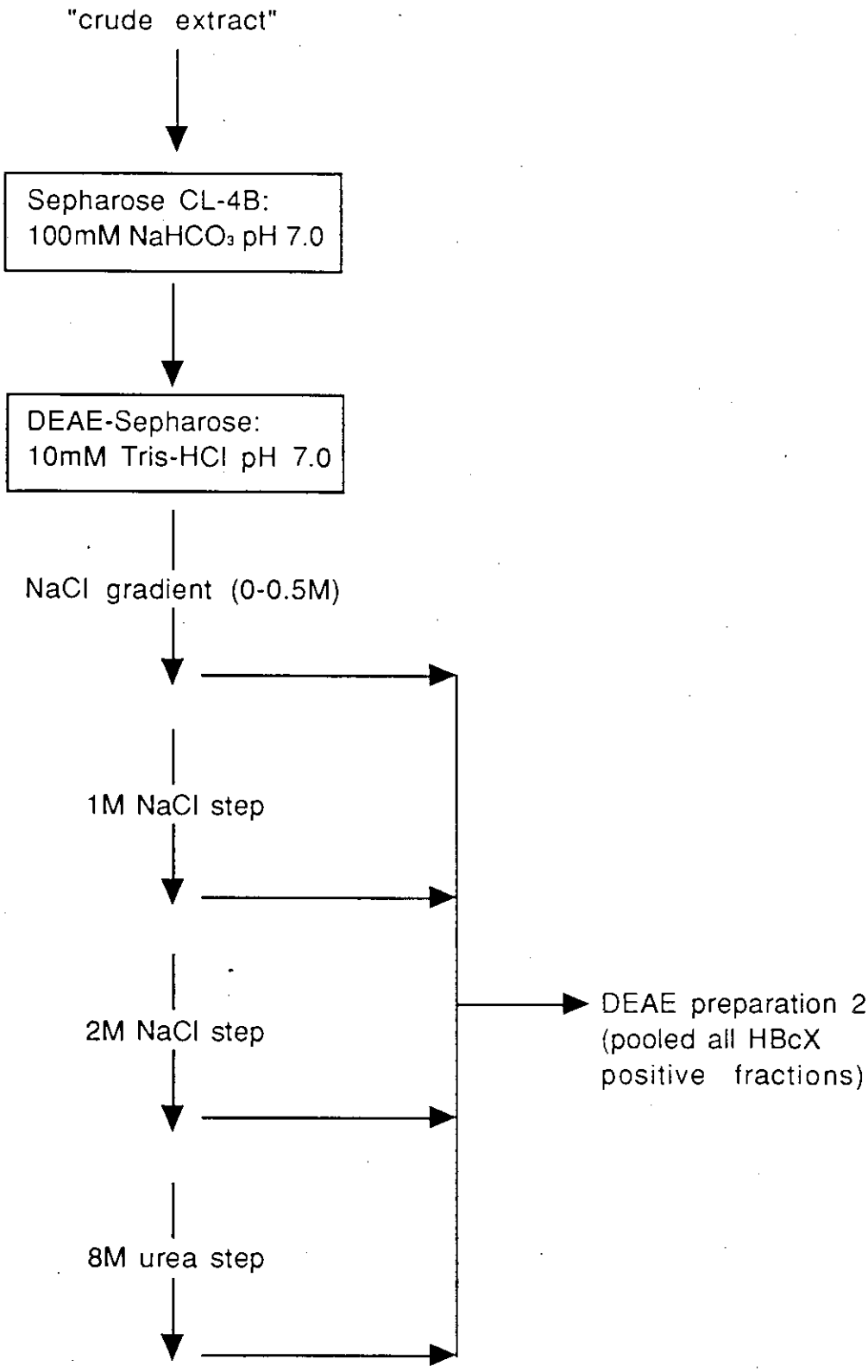
c) Large-scale preparation 2.

Purification steps used in this preparation are diagrammed in figure 2.2.

E. coli RB791 harbouring plasmid pHBcX were grown and induced as in large-scale preparation 1 (above). Cells were lysed as above except that DTT was added to 10mM immediately following crushing of cells in the French press, and DTT was present at that concentration in all subsequent solutions. Crude extract was prepared as described above, the preparation was split into two equal portions, and each half was fractionated by gel filtration through the column of Sepharose CL-4B column as described above. Fractions from each half containing HBcX were combined separately and fusion protein was sedimented as above. The protein pellets were each resuspended in 1.25 ml of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF, and each half was fractionated by

FIGURE 2.2 Scheme for purification of HBcX. Large-scale preparation 2.

Column chromatography stages of purification are indicated in boxes along with the buffer used for equilibration of each column. Details of the purification procedure are provided in the text (section 2B.10.10c).


"crude extract"
Sepharose CL-4B:
100mM NaHCO3 pH 7.0
DEAE-Sepharose:
10mM Tris-HCl pH 7.0
NaCl gradient (0-0.5M)
1M NaCl step
2M NaCl step
8M urea step
DEAE preparation 2
(pooled all HBcX
positive fractions)

chromatography through the DEAE-Sepharose column as described above. The positive fractions were combined, fusion protein was sedimented as above, and the protein pellet was resuspended in 200 μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

Additional protein was eluted from the DEAE-Sepharose column with steps of 100 ml of 1M NaCl, 100ml of 2M NaCl, and 100ml of 8M urea (all solutions were prepared in 10mM Tris-HCl pH 7.0). Fractions of eluate from each step containing HBcX were combined separately. Pooled fractions containing urea were dialysed against three changes of 1 litre each of 10mM Tris-HCl pH 7.0. Fusion protein was sedimented as above and each protein pellet was resuspended in 200μl of 10mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

After analysis by PAGE in 12.5% acrylamide gel, all of the protein preparations eluted from the DEAE-Sepharose column were combined, fusion protein was sedimented as above, and the protein pellet was resuspended in 150 μl of 10 mM Tris-HCl pH 7.0 containing 0.1mM PMSF.

2B.10.11 Preparation of crude protein extract from mammalian cells in culture

Confluent monlayers of mammalian cells were washed and scraped from the plate as described for RNA preparation (section 2B.2.3a). The cell pellet was resuspended in 1 ml of PBS containing 0.1mM PMSF and transferred to a microcentrifuge tube. The cell suspension was frozen by incubation for 10 seconds in liquid nitrogen and then thawed by incubation for 3 minutes at 37°C. The freeze-thaw cycle was repeated 2 more times, and cellular debris was removed by centrifugation (17,000 x g) in a microcentrifuge for 10 minutes at 4°C. The supernatant was recovered and stored at -70°C.

2B.10.12 Immunoprecipitation of soluble protein

Protein A Sepharose beads (Sigma) (10% w/v) were swollen by incubation in 1 ml of PBS for 1 hour at 37°C. The swollen beads were sedimented from suspension by centrifugation (17,000 x g)in a microcentrifuge for 15 seconds at room temperature. Beads were washed 4 times in several volumes of PBS and finally resuspended (10% (w/v)) in PBS and stored at 4°C.

The method used to immunoprecipitate soluble protein was that described by Harlow and Lane (1988). The appropriate antiserum was added to protein solution at a dilution of 1:250, the solution was mixed by gentle inversion and incubated for 1 hour on ice. One-tenth volume of protein A Sepharose bead suspension was added and the mixture was incubated for 1 hour at 4°C with rotation. The beads were sedimented from suspension by centrifugation (17,000 x g) in a microcentrifuge for 15 seconds and were washed 3 times in 10 volumes of PBS. The washed beads were used directly in a protein kinase assay.

2B.10.13 Assay for protein kinase activity

10 x protein kinase buffer:

100mM Tris-HCl pH 7.0
100mM $MgCl_2$
100mM DTT
5% NP-40

Protein kinase assays were carried out in the following 50 μl reaction mixture.

5 μl 10 x protein kinase buffer
10 μl HBcAg (2 μg/μl, *E. coli* product; Biogen)
34 μl protein sample (either soluble protein, or protein immobilised on protein A Sepharose beads)
1 μl [γ-^{32}P]ATP (10 μCi/μl; Amersham)

As a positive control, the protein sample used was 0.5 μg of bovine heart protein kinase (catlytic subunit of cAMP-dependent protein kinase; Sigma) in 34 μl H_2O. As a negative control, H_2O was used in place of HBcAg and protein sample. The kinase reaction was carried out for 1 hour at 37°C after which 10 μl of sample was spotted onto each of two 6.25cm^2 DE81 filters. Filters were allowed to dry and were then washed in several changes 0.5M Na_2HPO_4 pH7.0 over a period of 1 hour as described by Maniatis *et al.* (1982). Filters were then washed for 1 minute in H_2O and for 1 minute in absolute ethanol and allowed to dry at 60°C. The amount of radioactive phosphate incorporated

into protein was assessed by counting the Cerenkov radiation in the ^{3}H-channel of a Beckman LS7000 liquid scintillation counter, and this value was compared to the number of counts obtained from an unwashed filter onto which 10 μl of the negative control reaction mixture was spotted.

Labelled proteins in the kinase reaction mixture were separated by PAGE in 12.5% acrylamide gel and visualised by autoradiography. 30 μl of 2 x PAGE sample buffer (section 2B.10.2) was added to the remaining 30 μl of reaction mixture, samples were incubated for 5 minutes at 100°C, and protein A Sepharose beads were sedimented by centrifugation (17,000 x g) in a microcentrifuge for 15 seconds at room temperature. Soluble parts of the samples were loaded onto a 12.5% acrylamide gel, and electrophoresis was carried out as described above (section 2B.10.2). Proteins were electroblotted onto a nitrocellulose membrane, and visualised by staining with amido black NB (Sigma) as described above (section 2B.10.5). X-ray film was exposed to the membrane in a light-proof cassette with intensifying screens (Dupont Cronex Lightning Plus) at -70°C. Exposure times are indicated in figure legends.

2B.10.14 Electron microscopy of HBcAg/HBxAg fusion protein

Protein samples were diluted 10-fold in H_2O and 1μl of diluted sample was placed on a 400 mesh copper electron microscope grid. 3 μl of 2% (w/v) sodium phospho-tungstate pH 7.0 was placed over the protien sample and excess liquid was drained off with blotting paper. Samples were viewed and photographed at 40,000 x magnification using a Siemens Elmiskop 101 electron microscope.

CHAPTER 3: The Immunological Response to HBxAg

3.1 Introduction

Viral infection elicits a humoral (B-cell) and cellular (T-cell) immune response from the host directed against the viral antigens. HBxAg itself has not been detected in serum from HBV infected individuals (Weber *et al.*, 1988), however, indirect evidence for the expression of HBxAg *in vivo* has been provided by the detection of antibodies specific for this antigen in patient sera (Kay *et al.*, 1985; Moriarty *et al.*, 1985; Elfassi *et al.*, 1986; Meyers *et al.*, 1986; Pfaff *et al.*, 1987; Koike *et al.*, 1988; Weber *et al.*, 1988; Katayama *et al.*, 1989; Levrero *et al.*, 1990b; Stemler *et al.*, 1990). Some reports have attempted to determine whether the production of antibodies to HBxAg is diagnostic for any of the variable clinical features of HBV infection.

The overall conclusion from the above mentioned studies is that antibodies directed against HBxAg are produced during HBV infection in only a fraction of hepatitis B patients. Meyers and his colleagues (1986) assayed patient sera for anti-HBxAg by the ability to immunoprecipitate a labelled *X*-gene product produced in *E. coli*. In a panel of 49 HBsAg-positive sera, antibodies to HBxAg were detected in only one sample, while in a separate panel of sera characterised as containing antibodies to HBeAg, 9/26 were also anti-HBxAg positive. The observed correlation between the presence of antibodies specific for HBxAg and for HBeAg was consistent with a limited study by Kay *et al.* (1985).

Serum from 254 hepatitis B patients was screened for antibodies directed against HBxAg using an ELISA in which a synthetic peptide (representing either amino acid residues 100-115 or 144-154 in the deduced HBxAg primary structure) was immobilised on the solid phase (Moriarty *et al.*, 1985). Thirty-five sera were found to contain antibodies which bound to one or both peptides including sera from asymptomatic carriers (4/68), chronic hepatitis patients (20/149), and patients with liver cirrhosis (3/19) or HCC (8/11) following chronic HBV infection. The high fraction of sera from HCC patients that were positive for anti-HBxAg suggested that this response may be a marker for the development of HCC.

Both of the above correlations were called into question by further investigations of the humoral immune response to HBxAg. Several studies have revealed a stronger correlation between the chronic active hepatitis (CAH) disease state and production of antibodies to HBxAg than that observed in the same studies in sera from patients with HCC (table 3.1a). Sera from patients with CAH that were positive for anti-HBxAg were

Table 3.1a: Correlation between CAH and anti-HBxAg

	A	B	C	Total
Disease state				
CAH	4/6	6/31	3/14	13/51
HCC	11/42	2/49	0/3	13/94

Results from three studies indicating the ratio of patient sera in which antibodies to HBxAg were detected to the total number of sera examined. A: Pfaff *et al.*, 1987; B: Levrero *et al.*, 1990b; C: Elfassi *et al.*, 1986. Abbreviations: CAH = chronic active hepatitis; HCC = hepatocellular carcinoma.

Table 3.1b: Correlation between the presence of HBeAg and anti-HBxAg

	A	B	C	Total
Serological marker				
HBeAg	3/4	5/6	3/3	11/13
anti-HBeAg	0/4	1/6	N.T.	1/10

Results from three studies indicating the ratio of patients that were anti-HBxAg positive and also positive for the serological marker indicated to the total number of patients that were anti-HBxAg positive. References are the same as for table 3.1a. N.T. = not tested.

predominantly negative for anti-HBeAg, but positive for the presence of HBeAg (table 3.1b). HBeAg in patient serum is a marker for ongoing viral replication (Overby *et al.*, 1983) suggesting that production of antibodies to HBxAg requires chronic exposure to replicating virus. Antibodies to HBxAg in serum from patients with acute infection were not detected in any of the studies described thus far.

The requirement for chronic exposure to HBV replication for seroconversion to anti-HBxAg positive was called into question by Weber *et al.* (1988). Sera were assayed for the presence of antibodies to HBxAg by immunoblot analysis with an *X*-gene product produced in *E. coli*. From a panel of ten HBsAg-positive sera, cross reacting antibodies were detected in patients suffering acute infection (1/3), late acute infection (3/3), and in one of two chronic carriers. (The disease state of the patient was undefined for two of the serum samples which were both negative for anti-HBxAg antibodies.) Positive sera all contained HBeAg but were negative for anti-HBeAg. In the same study, weekly serum

samples collected during acute infection of chimpanzees experimentally inoculated with HBV were assayed for the presence of anti-HBxAg. All four animals seroconverted to anti-HBxAg positive within five months post-infection, but the response was transient, lasting at most 70 days. None of the control animals vaccinated with HBsAg or HBcAg prior to challenge with HBV developed antibodies to HBxAg. The occurrence of an immune response to HBxAg during acute infection has been confirmed by Stemler *et al.* (1990). However, the authors note that they found lower titres of anti-HBxAg antibodies in serum from patients experiencing acute infection compared to those with chronic HBV infection.

The detection of antibodies to HBxAg in serum from patients manifesting a number of disease states associated with HBV infection in both chimpanzees and humans indicates that the viral gene product is expressed and presented in an immunogenic form during various clinical manifestations of viral infection. The low proportion of HBV infected individuals producing antibodies to HBxAg may be due to the low amount of antigen produced by infected cells. *X*-gene specific transcripts have not been detected in HBV infected liver (Cattaneo *et al.*, 1984; Will *et al.*, 1987; Su *et al.*, 1989), and HBxAg has not been detected in serum from infected patients (Weber *et al.*, 1988). A consequence of intracellular compartmentation of HBxAg may be that it is only presented to the immune system upon liver cell necrosis, and higher titres of anti-HBxAg observed in sera from chronic hepatitis patients support the hypothesis that ongoing hepatocytolysis is necessary for presentation of HBxAg. Another factor in the low proportion of HBV infected individuals who produce antibodies specific for HBxAg may be the cellular codon usage demonstrated for this particular antigen of HBV (Miller and Robinson, 1986). If a distant cellular homolog is expressed an individual may develop tolerance to the viral antigen of similar shape.

Wu and her colleagues have recently reported that HBxAg is a component of the HBV virion. The ability of B cells to function as antigen presenting cells has been demonstrated by Lanzavecchia (1985), therefore a B-cell binding to and internalising the HBV virion could present peptide fragments of HBxAg in association with MHC class II antigens, thereby stimulating T-helper cells with HBxAg-specific receptors. The T-helper cell population thus stimulated may enhance the HBxAg specific B-cell response upon binding of antigen to the B-cell (see section 1.3.2 for a more detailed outline of this mechanism).

The aim of this part of my investigation was to begin to dissect the antigenic

determinants on HBxAg which bind antibodies produced during HBV infection or those raised in a rabbit inoculated with a denatured form of HBxAg expressed in *E. coli*.

3.2 Results

3.2.1 Plasmid constructions

Pugh *et al.* (1986) described synthesis in *E. coli* of HBxAg encoded by plasmid pBBX containing HBV sequences 2682-3182/1-88 inserted at the *Bam*HI site of plasmid pCL19ΔY (Zabeau and Stanley, 1982). pBBX encodes a fusion protein of 153 amino acids in which the 9 amino-terminal residues of HBxAg are replaced with the first 8 amino-terminal residues of λcro protein. Various segments of the X ORF contained in plasmid pBBX were inserted at the polylinker site in plasmid pEX2 or pEX3 (Stanley and Luzio, 1984). These two *E. coli* expression vectors contain a polylinker in a different translational reading frame at the 3′ end of a λ*cro*/*lac*I/*lac*Z fusion gene (figure 3.1). Transcription is under control of the bacteriophage λ right promoter and is heat inducible in *E. coli* cells (for example, strain NF1) harbouring a λ prophage encoding a temperature sensitive mutant c1 repressor protein. A large proportion of the resulting fusion protein is composed of *E. coli* polypeptide sequences yielding a stable product accounting for up to 30% of protein in crude cell-extracts. In addition, sequences fused to the carboxyl-terminus of β-galactosidase using the pEX system have been shown to maintain their antigenicity in immunoblot analysis (Stanley and Luzio, 1984; McGlynn and Murray, 1988).

HBV DNA inserts in plasmid pEX are shown in figure 3.2, and the corresponding fusion proteins encoded by these plasmid constructions are shown in figure 3.3. Plasmid pXA was constructed to express in the pEX system all of the HBxAg coding sequence (amino acid residues 10-154) contained in plasmid pBBX. Plasmid pEX3 was linearised by digestion with *Sal*I, filling in cohesive ends with DNA polymerase I (Klenow fragment), and digestion with *Bam*HI. HBV sequences (2683-3158) were isolated from plasmid pBBX by digestion with *Bam*HI and *Alu*I, and the resulting 475 bp DNA fragment was ligated to the prepared pEX vector DNA. Recombinant plasmids were selected following transformation of *E. coli* strain MC1061(λ) with DNA in the ligation mixture. These cells contain a wild type λ prophage and cells harbouring a pEX based plasmid construction may be grown at 37°C while maintaining repression of expression from the λ right promoter contained in the plasmid. Recombinant plasmid DNA was purified from *E. coli*

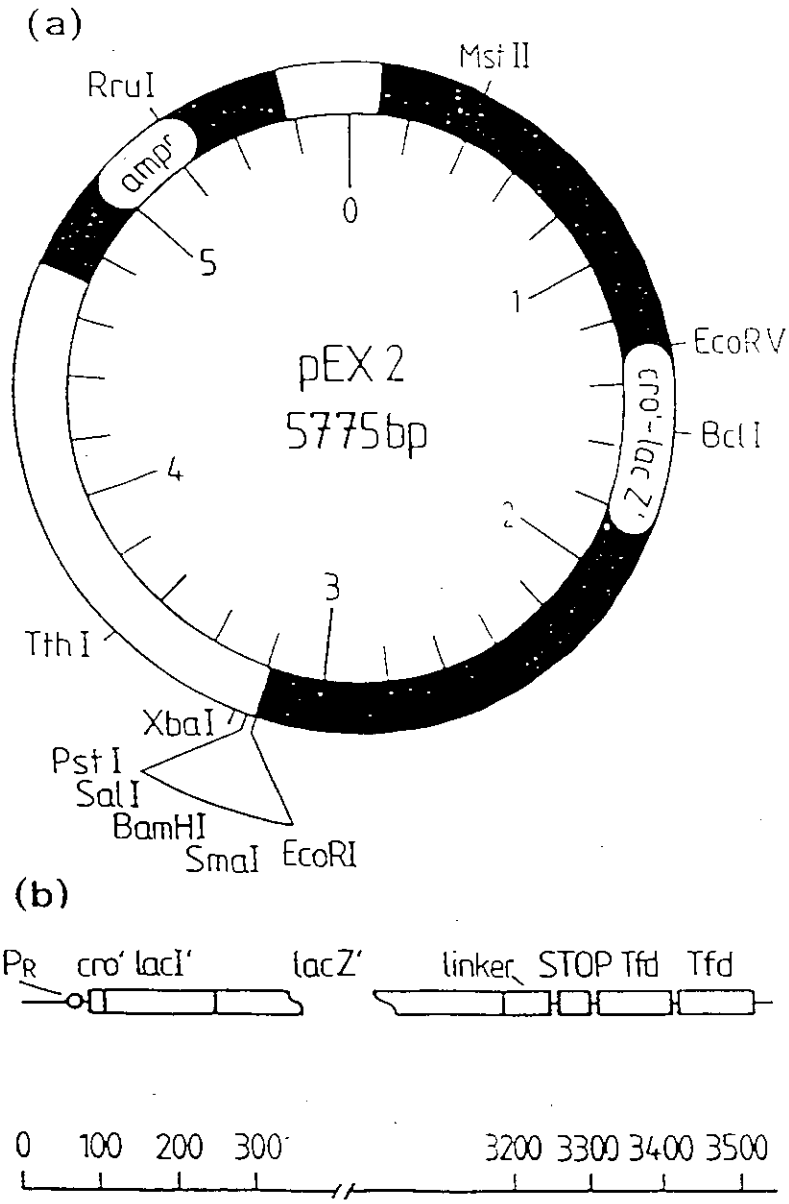


FIGURE 3.1: pEX vector

A) Map of plasmid pEX2 showing unique restriction sites.

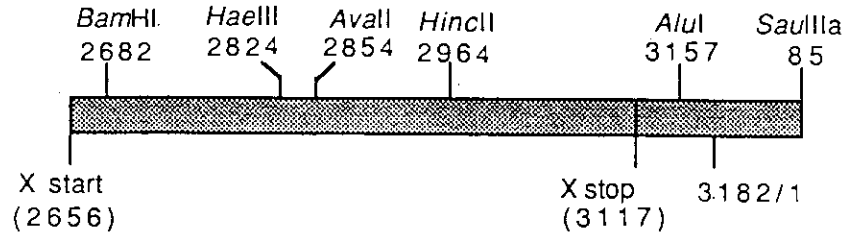
B) Organisation of the fusion gene and of translational and transcriptional control elements contained in the plasmid. A detailed description is provided in section 2A.3.2. Tfd = transcription terminator fragment from bacteriophage fd.

From Stanley and Luzio, 1984.

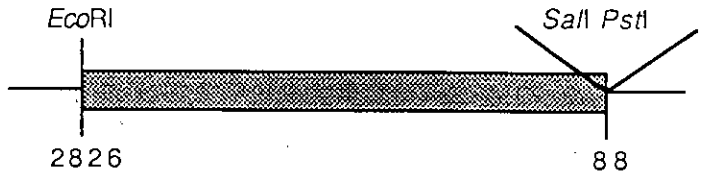
FIGURE 3.2

Top: X ORF and 3´ flanking sequence in HBV subtype *adyw* used in this study. Restriction endonuclease cleavage sites used for subcloning fragments of the X ORF into plasmid pEX are indicated. Numbers under restriction sites represent the position of the first nucleotide of the recognition sequence. The positions of the first and last nucleotides of HBxAg coding sequence are indicated.

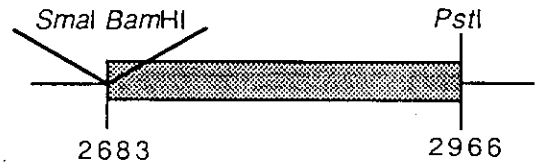
Below: Fragments of the X ORF contained in plasmids encoding β-galactosidase/HBxAg fusion proteins. Plasmids are shown in the order in which their products appear in figure 3.4. Restriction endonuclease cleavage sites flanking the insert of HBV DNA are indicated. The numbers represent the first and last nucleotides of HBV sequence contained in the recombinant plasmids. (Construction of plasmids is described in detail in section 3.2.1)



pXM3

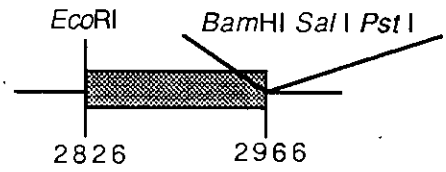


pX5M

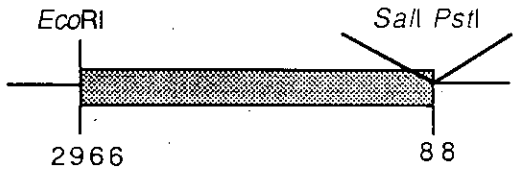


pXA

pXM



pX3



pX5

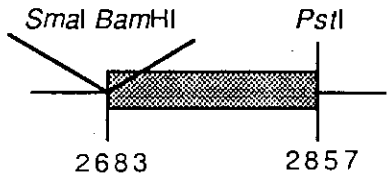
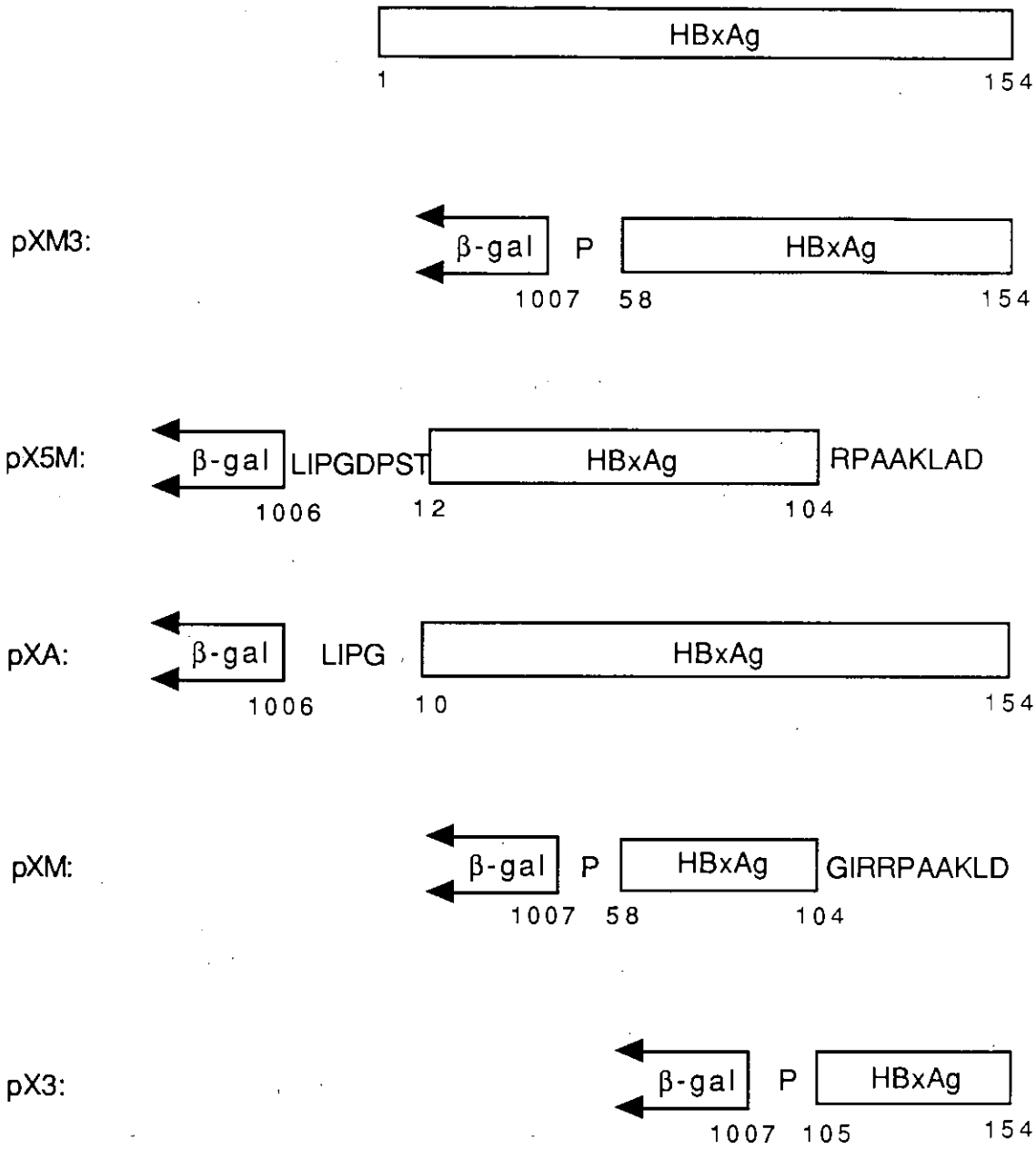


FIGURE 3.3

Top: Open box representing the complete amino acid sequence of HBxAg.

Below: Predicted amino acid sequences in the single letter code of β-galactosidase/HBxAg fusion proteins. Names of plasmids encoding the proteins are at left in the order in which their products appear in figure 3.4. "β-gal" represents the protein encoded by the λ*cro*/*lac*I/*lac*Z fusion gene contained in the pEX vector with the number representing the amino acid position in native β-galactosidase. Numbers under HBxAg boxes indicate the positions of the amino acids in the native protein.



high yield

low yield as with pSE X-5

MC1061(λ) cells and used to transform *E. coli* NFI to allow heat inducible expression of the fusion protein encoded by the plasmid. This transformation procedure was used for isolation of all the following pEX constructions. DNA sequence determination confirmed that the junction of the 3´ end of the *lac*Z gene and the 5´ end of the HBV DNA insert in plasmid pXA was as expected and the open reading frame was maintained through the *X*-gene stop codon. However, a *Bam*HI site was revealed at the 3´ junction of HBV sequence and vector sequence. This most likely arose from a failure of the *Sal*I digestion of the pEX vector DNA resulting in a linearised plasmid with *Bam*HI cohesive ends. In this model, proper ligation of the *Bam*HI cohesive ends at the 3´ end of the *lac*Z gene and the 5´end of the HBV DNA insert occurred *in vitro*. Upon transformation of *E. coli* MC1061(λ) the 5´ overhang of the remaining *Bam*HI cohesive end was repaired by host cell DNA polymerase and this blunt end was ligated to the blunt end created by digestion of HBV DNA with *Alu*I.

Plasmid pXM3 contains the coding sequence for the carboxy-terminal two thirds of HBxAg (amino acid residues 58-154) fused to the 3´ end of the *lac*Z gene and was constructed as follows: plasmid pEX2 was linearised by digestion with *Bam*HI and *Sma*I. HBV sequences (2826-3182/1-88) were isolated from plasmid pBBX by digestion with *Hae*III and *Sau*IIIa, and the resulting 444 bp DNA fragment was ligated to the prepared pEX vector DNA. The integrity of the vector/insert junctions and of the X ORF was confirmed by DNA sequence determination.

Plasmid pX5M contains the coding sequence for the amino-terminal two thirds of HBxAg (amino acid residues 12-104) fused to the 3´ end of the *lac*Z gene and was constructed as follows: HBV sequences (2683-2966) were isolated from plasmid pBBX by digestion with *Bam*HI and *Hinc*II, and the resulting 283 bp DNA fragment was ligated to plasmid pEX3 DNA prepared as described for construction of pXA. DNA sequence determination revealed that the vector DNA had only been digested with *Sal*I and this site had been rendered blunt by the action of DNA polymerase I (Klenow fragment). It was determined that this site had been ligated to the *Bam*HI site at the 5´ end of the HBV DNA insert whose cohesive end had been repaired. The *lac*Z ORF was maintained at this junction resulting in the loss of only two codons from the 5´ end of the desired X ORF insert. The integrity of the X ORF and 3´ insert/vector junction was confirmed by DNA sequence determination.

Plasmid pXM contains the coding sequence for the middle third of HBxAg (amino acid residues 58-104) fused to the 3´ end of the *lac*Z gene and was constructed as follows:

plasmid pEX2 was linearised by digestion with *Eco*RI and *Sma*I. The HBV DNA insert in plasmid pXM3 was isolated by digestion with *Eco*RI and *Xba*I, which cuts 40 nucleotides downstream of the polylinker site in the pEX vector sequence. The resulting 502 bp DNA fragment was digested with *Hinc*II, and the resulting 140 bp DNA fragment (HBV nucleotide positions 2826-2966) was ligated to the prepared vector DNA. The integrity of the vector/insert junctions and of the X ORF was confirmed by DNA sequence determination.

Plasmid pX3 contains the coding sequence for the carboxy-terminal third of HBxAg (amino acid residues 105-154) fused to the 3´ end of the *lac*Z gene and was constructed as follows: plasmid pEX2 was prepared as described for construction of pXM3. The HBV DNA insert in plasmid pXM3 was excised by digestion with *Eco*RI and *Xba*I. The resulting 502 bp DNA fragment was digested with *Hinc*II and *Sau*IIIa, and the resulting 304 bp fragment (HBV nucleotide positions 2966-3182/1-88) was ligated to the prepared vector DNA. The integrity of the vector/insert junctions and of the X ORF was confirmed by DNA sequence determination.

Plasmid pX5 contains the coding sequence for the amino-terminal third of HBxAg (amino acid residues 12-67) fused to the 3´ end of the *lac*Z gene and was constructed as follows: plasmid pEX2 was prepared by digestion with *Sal*I, filling in cohesive ends with DNA polymerase I (Klenow fragment), and digestion with *Sst*I which cuts following the codon for β-galactosidase residue 650 in the pEX vector sequence. HBV sequences (2683-2857) and 1 kb of the *lac*Z gene were isolated from plasmid pX5M by digestion with *Ava*II, filling in cohesive ends with DNA polymerase I (Klenow fragment) and digestion with *Sst*I. The resulting 1.2 kb DNA fragment was ligated to the 4.3 kb vector DNA fragment. DNA sequence determination confirmed that the X ORF, which had been contained in plasmid pX5M, was deleted to codon 67 and the fusion protein coding sequence terminated in the vector sequence downstream of the 3´ insert/vector junction.

3.2.2 Antigenicity of HBxAg fragments fused to β-galactosidase

β-galactosidase/HBxAg fusion proteins were expressed by heat induction of *E. coli* NF1 cells harbouring one of the recombinant plasmids described above. Total protein from a panel of induced cells was size fractionated by SDS-PAGE in a 7% acrylamide gel and was detected by staining with Coomassie Brilliant Blue (figure 3.4a). The size of the fusion proteins was in close agreement with that predicted from the constructions.

FIGURE 3.4: Antigenicity of β-galactosidase/HBxAg fusion proteins produced in *E. coli*

Extracts of *E.coli* cells alone (NF1), cells harbouring the vector plasmid (pEX) and cells harbouring plasmids encoding β-galactosidase/HBxAg fusion proteins (pXM3, pX5M, pXA, pXM, pX3, pX5) were fractionated by SDS-PAGE in a 7% acrylamide gel.

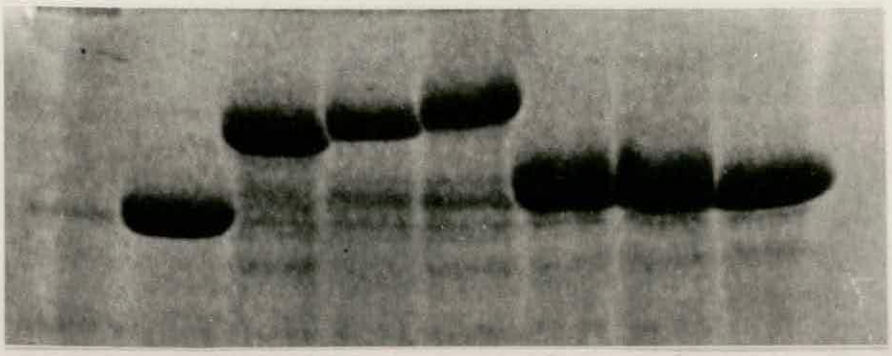
A) Coomassie Blue stain of acrylamide gel.

B) Proteins fractionated by SDS-PAGE were transferred to nitrocellulose membrane and incubated with serum from chimpanzee Peter (section 2A.5) diluted 1:200. Antibodies which bound proteins on the membrane were detected by incubation with goat anti-human IgG antiserum conjugated to alkaline phosphatase and subsequent incubation with a colour producing substrate as described in section 2B.10.8. Densitometric analysis of the resulting bands was done on the Shimadzu Dual Wavelength Cromato Scanner, Model CS-930. The numbers below each lane represent the peak reflective intensities obtained.

C) Immunoblot analysis as in "B" using a 1:5000 dilution of rabbit 98 anti-HBxAg serum (section 2A.5) for the primary incubation and goat anti-rabbit IgG serum for the secondary incubation. Rabbit 98 antiserum had been preadsorbed against a lysate of *E. coli* strain NF1 producing the pEX vector fusion protein (section 2B.10.7)

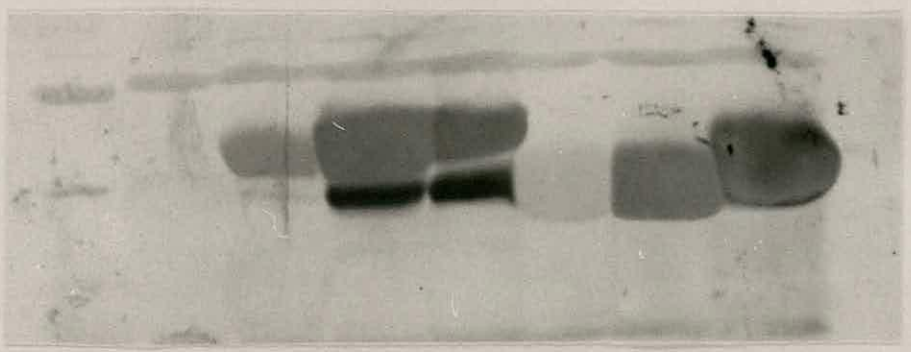
A. Coomassie

NF1 pEX pXM3 pX5M pXA pXM pX3 pX5



B. Chimpanzee

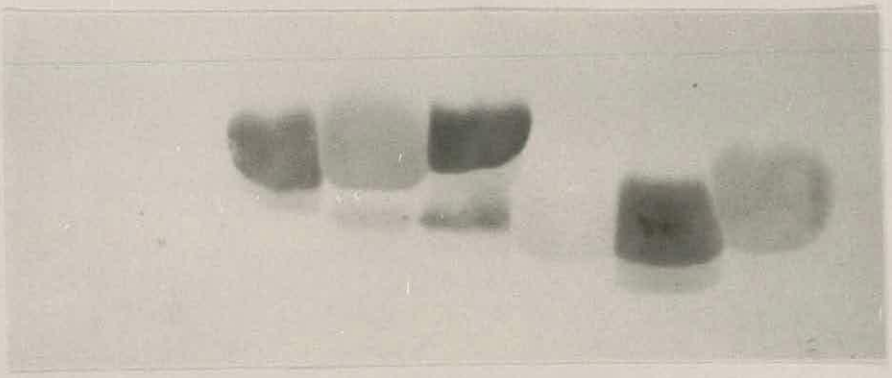
NF1 pEX pXM3 pX5M pXA pXM pX3 pX5



.93 .253 .333 .357 .97 .274 .379

C. Rabbit

NF1 pEX pXM3 pX5M pXA pXM pX3 pX5



.17 .246 .140 .310 .62 .309 .141

The antigenicity of the fusion proteins was assessed by immunological detection of proteins transferred to a nitrocellulose membrane after SDS-PAGE. A rabbit antiserum was raised by Pugh *et al.* (1986) against a denatured form of HBxAg produced in *E. coli* harbouring plasmid pBBX and purified by SDS-PAGE (section 2A.5). The antiserum was pre-adsorbed against a lysate from *E. coli* NF1 cells transformed with plasmid pEX2 in which expression of the pEX vector fusion protein had been induced by incubation at 42°C. This procedure removed antibodies that cross-reacted with endogenous *E. coli* NF1 proteins and with the β-galactosidase fusion protein encoded by the pEX vector. (The pEX vector protein contained the same eight amino acids of λ cro protein as the HBxAg-fusion protein with which the rabbit was inoculated.) Serum withdrawn from the rabbit prior to inoculation with HBxAg did not react with pEX vector protein or any of the fusion proteins (data not shown). The numbers in figure 3.4b,c represent the peak reflective intensities obtained by densitometric analysis of the bands on the membrane following immunoblotting. The pre-adsorbed rabbit antiserum did not react with pEX vector protein; it reacted strongly with antigenic determinants in the carboxy-terminal third of HBxAg, less strongly with those in the amino-terminal third of the viral antigen, and only minimally with those in the middle third of HBxAg when these sequences were presented fused to β-galactosidase (figure 3.4c; pX3, pX5 and pXM, respectively). The pattern of antigenicity for the carboxy- and amino-terminal thirds of the protein was repeated when the middle third of the antigen was included in proteins expressed from plasmids pXM3 or pX5M, respectively. Immunological detection revealed breakdown-products of some of the fusion proteins that retained reactivity with the anti-HBxAg antisera. It was not possible to assess the exact constitution of these products, therefor they were not included in the densitometric analysis.

The antigenic determinants recognised by anti-HBxAg antibodies produced during acute infection with HBV were determined as above for serum withdrawn from chimpanzee Peter 184 days after experimental infection. It had been shown previously by immunoblotting that this serum contained antibodies that reacted with HBxAg produced in *E. coli* encoded by plasmid pBBX (Weber *et al.*, 1988). Serum withdrawn prior to infection did not react with any of the β-galactosidase/HBxAg fusion proteins (data not shown). The anti-HBxAg-positive serum showed virtually no reactivity with the pEX vector protein, but reacted strongly with fusion proteins containing either the carboxy- or amino-terminal third of HBxAg (figure 3.4b; pX3 or pX5, respectively). As observed with the rabbit antiserum, reactivity with the middle third of HBxAg was weak

(pXM), and when this segment was included with either the carboxy-terminal third (pXM3) or amino-terminal third (pX5M) of the antigen it did not alter the pattern of reactivity observed when these segments were presented on their own.

3.3 Discussion

The presence of antibodies directed against HBxAg in the rabbit anti-serum and the chimpanzee serum indicates that both denatured and native HBxAg (respectively) are immunogenic. The polyclonal response elicited in both cases contained antibodies that reacted with the denatured β-galactosidase/HBxAg fusion proteins immobilised on nitrocellulose membrane following SDS-PAGE. The titre of antibodies in the chimpanzee serum recognising denatured antigenic determinants on HBxAg was much lower than in the rabbit anti-serum raised against a denatured form of the antigen. In order to obtain comparable signals the chimpanzee serum was used at a dilution of 1:200 while the rabbit anti-serum was used at a dilution of 1:5000.

Antibodies elicited by a native protein are directed against two classes of antigenic determinants (reviewed by Benjamin *et al.*, 1984). Conformational determinants depend on the native spatial arrangement of two or more peptide regions in the protein and it is improbable that they would be maintained following denaturation of a protein for SDS-PAGE. Continuous determinants depend only on the amino acid sequence of a peptide segment. Antibodies developed against the native antigen will be directed against the accessible surface which can combine with appropriate antibodies displayed by the B-cell repertoire. Space filling models of globular proteins indicate that very few continuous stretches of residues are present at the surface of a protein (Barlow *et al.*, 1986; Van Regenmortel, 1989). Therefore, the majority of antibodies raised against a native protein will depend on conformation for binding, accounting in part for the low titre of antibodies in the chimpanzee serum reacting with the denatured antigens used in the assay in this study. Other factors contributing to the low titre of antibodies to HBxAg in the chimpanzee are the low level of HBxAg produced during infection and the fact that only a fraction of the amount produced is presented to the immune system upon hepatocyte necrosis.

Both chimpanzee and rabbit sera gave differential binding to the various segments of HBxAg expressed in the β-galactosidase/HBxAg fusion proteins. This differential is most likely due to the presence in the serum of a greater number of antibodies directed against

a particular antigenic determinant rather than antibodies of greater affinity remaining bound to the protein immobilised on the membrane. Immunoblotting does not require high affinity antibodies due to the high local concentration of antigen on the membrane. Antibodies can bind through both Fab arms, and, if dislodged, low affinity antibodies have the opportunity to bind adjacent sites (Harlow and Lane, 1988).

Both rabbit and chimpanzee sera reacted weakly with the middle third of HBxAg. In the case of the anti-native HBxAg chimpanzee serum, a weak reaction may indicate that this segment of HBxAg is buried in its native conformation and is not accessible for binding to appropriate antibodies displayed by the B-cell repertoire. That is, an antigenic response is not seen because the region is not immunogenic in the animal. The pattern of antigenic determinants observed with the chimpanzee serum was in good agreement with a computer prediction of antigenic sites in HBxAg (figure 3.5) (Jameson and Wolf, 1988). The algorithm used in the program calculates an antigenic index which is an indication of potentially exposed surface peaks of a native protein based on its amino acid sequence. It takes into account parameters that have been shown to correlate with sites of antigenic determinants including hydrophilicity (Hopp and Woods, 1981), flexibility (Westhof *et al.*, 1984) and probability of situation at the surface of the protein (Emini *et al.*, 1985). The computer program also considers predictions of secondary structure such as β-turns (Chou and Fasman, 1978; Garnier *et al.*, 1978) which would contribute to flexibility.

It is interesting that the same region of HBxAg that reacted weakly with the chimpanzee antiserum also reacted poorly with the rabbit antiserum. Features of secondary and tertiary structure of the immunogen should not be a factor in this case, as it was denatured by boiling in SDS. Antibody production would have been determined by the immune regulatory mechanisms in the host whose response cannot be predicted for a protein of unknown form.

From the single humoral response to native antigen investigated in this study, it is not possible to generalise about regions of HBxAg that may be immunodominant. The humoral response to particular portions of an antigen vary markedly among individuals of a particular species and one needs to evaluate a consensus response from several samples before speculating about immunodominance (Geysen *et al.*, 1987). Such a study has been reported recently for HBxAg (Stemler *et al.*, 1990).

Stemler and her colleagues investigated the distribution of antibodies directed against various segments of HBxAg in 50 sera from HBV patients previously shown to contain

FIGURE 3.5: Secondary structure and antigenicity prediction for HBxAg.

The secondary structure prediction is that of Chou and Fasman (1978) and antigenicity was computed using an algorithm developed by Jameson and Wolf (1988). Both predictions were plotted using the University of Wisconsin Genetics Computer Group (UWGCG) "Peptidestructure" and "Plotstructure" programs (Devereux *et al.*, 1984). Features of predicted secondary structure are:

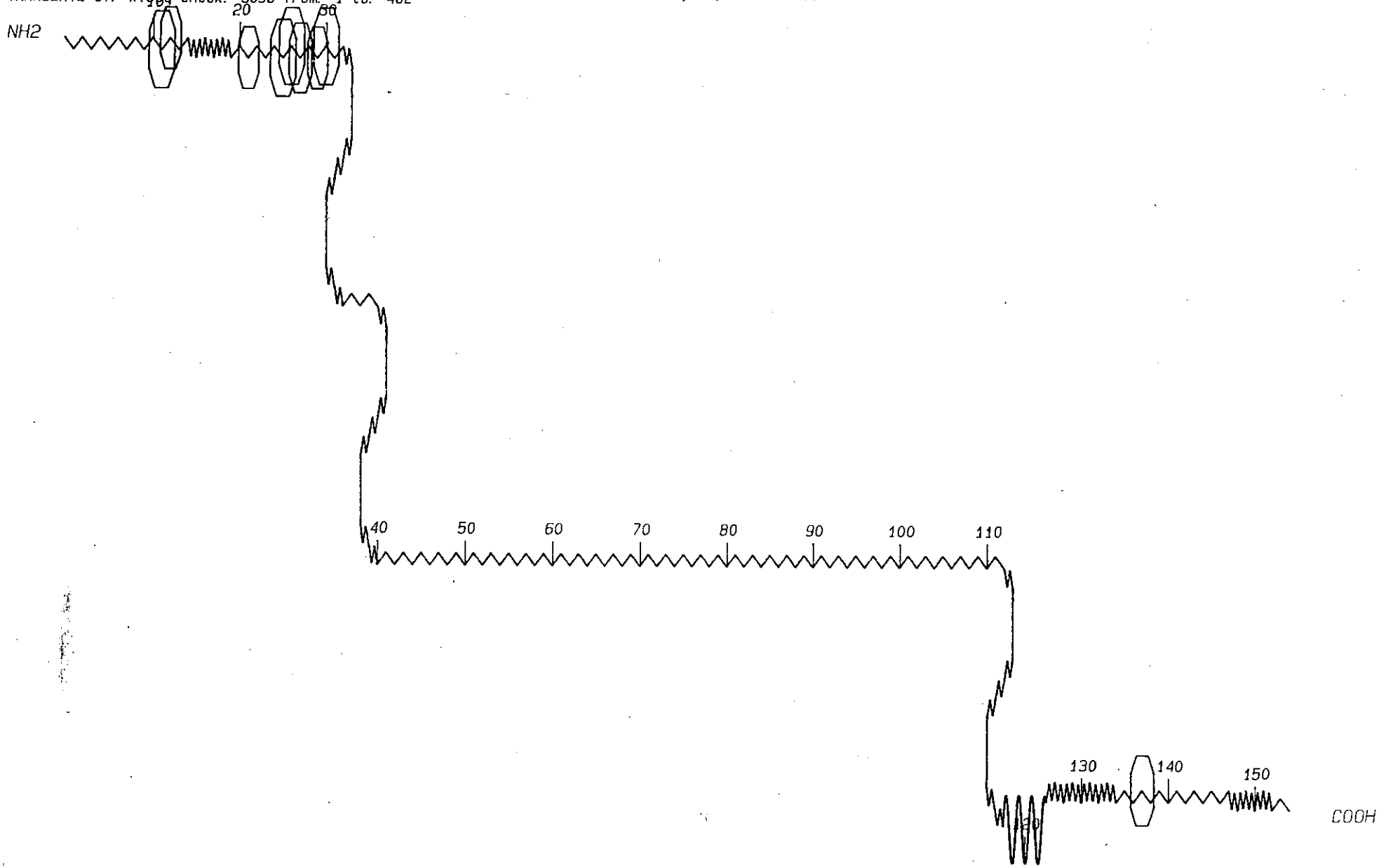
 - β-turns

 - α-helix

 - β-sheets

 - random coil

The degree of antigenicity is indicated by the size of the polygon positioned on the polypeptide. The threshold value of 1.2 for the antigenic index is the default value contained in the "Plotstructure" program.


PLOTSTRUCTURE of: x.pep ck: 1110
TRANSLATE of: x.seq check: 3098 from: 1 to: 462
Chou-Fasman Prediction
January 10, 1991 11:01
0 Antigen.Index >= 1.2
NH2
10
20
30
40
50
60
70
80
90
100
110
130
140
150
COOH

antibodies specific for HBxAg. Antigenicity was determined by immunoblotting for a panel of 17 proteins produced in *E. coli* comprising various segments of HBxAg fused to a portion of the replicase gene of bacteriophage MS2. Nearly all of the sera (43/50) reacted with 3 of the fusion proteins, indicative of an immunodominant region contained within amino acids 90-102. 21 of 50 sera reacted with fusion proteins presenting an animo-terminal antigenic determinant (a.a. 33-67), while reactivity with the carboxy-terminal third of HBxAg was rare with only 2 of 50 sera reacting with an antigenic determinant at amino acids 144-154. An immunodominant determinant in the region of amino acids 85-110 was confirmed by ELISA. A panel of 38 anti-HBxAg positive sera was screened for reactivity with overlapping synthetic peptides of 15 residues representing the entire HBxAg primary structure. Antigenic determinants in peptides comprising both the amino- and carboxy-terminal thirds of the antigen (or both) were also detected by this analysis in greater than 50% of the sera assayed. The immunoblot presented by Stemler *et al.* for one of the two sera reactive with the carboxy-terminal determinant shows weak reactivity with the immunodominant region proposed in their analysis. The pattern of reactivity for this serum with amino- and carboxy-terminal determinants is similar to that detected with the chimpanzee serum in this study which may have been a rare observation.

The study of antigenic determinants on a protein molecule provides a database of information about the nature of the mammalian humoral immune response. This knowledge is invaluable for predicting the immune response to other antigens for vaccine development or in the investigation of autoimmune disease.

CHAPTER 4: HBxAg Modulates Expression of HBsAg

4.1 Introduction: General Features of Transactivation by HBxAg

The ability of HBxAg to transactivate a wide array of targets has been investigated in several recent studies (table 4.1; references in Appendix II). Most of these investigations have been carried out by transient expression of the bacterial chloramphenicol acetyl transferase (CAT) gene in mammalian cells under control of a potential target promoter or promoter/enhancer complex. In many studies, HBxAg was supplied in *trans* by cotransfecting a second plasmid containing the HBxAg coding sequence under control of its own promoter (Twu and Schloemer, 1987; Seto *et al.*, 1988; Spandau and Lee, 1988; Twu and Robinson, 1989; Twu *et al.*, 1989a,b; Aufiero and Schneider, 1990; Hu *et al.*, 1990) or under control of a heterologous promoter, most commonly the SV40 early promoter/enhancer complex (Twu and Schloemer, 1987; Seto *et al.*, 1988; Spandau and Lee, 1988, Wollersheim *et al.*, 1988; Koike *et al.*, 1989; Seto *et al.*, 1989; Twu and Robinson, 1989; Faktor and Shaul, 1990; Rossner *et al.*, 1990; Seto *et al.*, 1990; Unger and Shaul, 1990, Zhou *et al.*, 1990).

The transactivating potential of HBxAg is maintained in the context of the whole HBV genome either expressed transiently in hepatoma cells (Colgrove *et al.*, 1989; Koike *et al.*, 1989) or integrated into hepatoma cell chromosomes following stable transfection (Zahm *et al.*, 1988; Siddiqui *et al.*, 1989; Twu and Robinson, 1989; Zhou *et al.*, 1990). In one example (Twu and Robinson, 1989), transactivation of the HIV1 LTR fused to the CAT gene was observed in HepG2 cells stably transfected with HBV DNA and producing virus providing further evidence that HBxAg is expressed during HBV replication.

HBxAg coding sequences isolated from HBV integrates associated with HCC (Wollersheim *et al.*, 1988) or chronic hepatitis (Takada and Koike, 1990) maintain the capacity to transactivate. The implications of this function on the correlation between HBV infection and the development of neoplasia will be considered in Chapter 6.

HBxAg produced in *E. coli* and administered to cultured mammalian cells by protoplast fusion (Twu *et al.*, 1990) or by direct application or "scrape-loading" of the purified product (Jameel *et al.*, 1990) can transactivate an integrated DNA construction containing the HIV1 LTR fused to the CAT gene. Purified, *E. coli*-derived HBxAg (Wu *et al.*, 1990) and HBxAg produced in hepatoma cells (Aufiero and Schneider, 1990) retain their transactivating capacity in transcription assays *in vitro*.

The transactivating function of HBxAg is conserved among all the mammalian hepadnaviruses (Colgrove *et al.*, 1989; Seto *et al.*, 1989). Interestingly, Colgrove and his colleagues (1989) note that the DHBV genome does not encode a polypeptide with the

Table 4.1: Transactivation by HBxAg

The targets that have been examined for susceptibility to transactivation by HBxAg are shown along with the cell lines in which experiments were conducted. References are listed in Appendix II. "Low" indicates a low level of transactivation observed compared to transactivation of other regulatory regions in the same study, or, when considering a mutated regulatory region, compared to transactivation of the parent construction.

[a] Tandem NF-κB binding sites derived from the HIV1 LTR were fused to the tk promoter fused to the CAT gene.
[b] reference 18: NF-κB sites as in "a", but containing point mutations; reference 13: HIV1 LTR fused to the CAT gene containing point mutations in the NF-κB binding sites, or containing a deletion from the 5′ end of the LTR through the NF-κB binding sites; reference 10: HIV1 LTR fused to the CAT gene containing a deletion as in reference 13.
[c] SV40 early promoter plus 32 nucleotides of the SV40 enhancer containing a binding site for transcription factor AP-1 fused to the CAT gene.
[d] HBV enhancer (nucleotide positions 2351-2531) containing point mutations or a small deletion in the EF-C binding site (see section 1.4.3a) was fused to the SV40 early promoter fused to the CAT gene.
[e] H-2K^b promoter containing point mutations in the NF-κB binding site and fused to the CAT gene.

Abbreviations:

enh -	enhancer
prom.-	promoter
mut. -	mutant
mur. -	murine

for all others, see abbreviations list

	Transactivation		No Transactivation	
Transactivation Target	**Cells**	**References**	**Cells**	**References**
HBV regulatory sequences				
HBV core promoter	HepG2 (low)	13	PLC/PRF/5	14
	HuH7 (low)	2		
HBV core promoter / enh	PLC/PRF/5	14	CV1	14
	HuH7	2	HuH7	6
	HepG2	6,13	HepG2	4a
HBV preS2/S promoter / enh	HepG2	6,9		
	HuH7	2,9	HuH7	6,24a
HBV preS1 promoter / enh	HepG2	6,9		
	HuH7	9	HuH7	6,24a
	Hep3B	8a		
HBV X promoter / enh	CCl13	22	Vero	16
	HepG2	3,8,13,21	HuH7	6
	PLC/PRF/5	3		
	Sk-Hep1	3		
Other viral sequences				
Adenovirus E2 promoter			Vero	16
Ad2 VA1 mRNA	Chang liver	1		
Herpes simplex tk prom.	CCl13	24	HepG2	18
			Jurkat	10
HIV1 LTR	HepG2	13,17,18,19,20	HepG2	10
	Jurkat	10,11	HuH7	17
	H938 (T-cell)	20	PLC/PRF/5	11
	MT2 (T-cell)	17	Raji (Burkitt)	11
	U937 (T-lymphoma)	11	SupT1 (T-lymphoma)	11
	SW480 (colon)	11	U343MG (glioma)	11
	HFF (fibroblast)	11	YAC-1 (mur. lymphoma)	11
	U251MG (glioma)	11		
	HeLa	5,13,17	HeLa	10,11
	Vero	17	COS7	11
	CV1	11	CV1+Tag	11
	MouseL	11	NIH3T3	11
HIV1 NF-κB[a]	HepG2	18		
	Jurkat	10		
HIV1 NF-κB mutant[b]	Jurkat (low)	10	HepG2	13,18
HIV2 LTR	HepG2	8		

	Transactivation		No Transactivation	
HTLV1 LTR	HepG2	13	HepG2	17
	CCl13	22,24	Vero	16
HTLVII LTR	HepG2	13		
MMTV LTR	CCl13	22,24		
MSV LTR			Vero	16
RSV LTR	CV1	11,14	Jurkat	11
	CCl13	24	Vero	16
	HuH7	2		
	PLC/PRF/5	2		
SV40 early promoter / enh	Vero	24	Vero	16
	CV1	14	COS	14
	HepG2	1,6,13,15,17,24	CV1+Tag	14
	HeLa	24		
	CCL13	22,24		
	NIH3T3	6		
	Fischer Rat	24		
	PLC/PRF/5	2		
	Chang Liver	1		
SV40 early promoter	NIH3T3	6	HepG2	13
			PLC/PRF/5	3
SV40 early promoter / AP1[c]	CCl13	22	Vero	16
	NIH3T3	6	CV1	14
			PLC/PRF/5	3
SV40 early promoter / BK enh	Vero	16		
SV40 early prom. / HBV enh	HepG2	3,3a,8,13	CHO	21a
	PLC/PRF/5	3		
	Sk-Hep1	3		
SV40 early prom. / HBV enh (ΔEF-C)[d]			HepG2	3a
SV40 late prom. / HBV enh	HepG2	8		
Visna virus LTR			HepG2	17

Synthetic factor binding sites	Transactivation		No Transactivation	
AP-1	CV1	12		
AP-2	CV1	12	HepG2	18
AP-3			CV1	12
HBV E site	PLC/PRF/5	3		
HBV EP site			PLC/PRF/5	3
HBV UE2 site			PLC/PRF/5	3
NK-κB	HepG2 PLC/PRF/5	18 3		
Cellular promoter sequences				
α-1-antitrypsin promoter/ HBV enhancer	HepG2	7		
actin			H938 (T-cell) HepG2 CCl13	20 8,13 24
α-globin			PLC/PRF/5	3,21
β-interferon	Vero	16	HepG2	25
HLA-DR (MHC class II)	HepG2 HuH7	4 4		
metallothioneine	CCl13 (low)	24		
murine H-2K^b(MHC class I)	HepG2	25		
murine H-2K^b -NFκb mut.[e]			HepG2	25
c-*myc*	NIH3T3	6		
serum albumin			HepG2	4,9
tRNAala	Chang liver	1		

capacity to transactivate the Rous sarcoma virus (RSV) LTR in human hepatoma cells (HuH7), despite being able to replicate in these cells (HBxAg and GSHxAg were both active under these conditions). This result provides evidence against speculation that the DHBV genome may encode an "X-like" polypeptide within its core ORF (Feitelson and Miller, 1988; Feitelson *et al.*, 1990).

Transactivation by WHxAg and GSHxAg gives an initial indication of the sequences in HBxAg required for this function as the rodent antigens are missing 7 and 12 residues, respectively, from the carboxyl-terminus in comparison with HBxAg. Segments of HBxAg required for transactivating function have been analysed in several systems by deletion analysis (Faktor and Shaul, 1990; Levrero *et al.*, 1990a; Takada and Koike, 1990; Unger and Shaul, 1990; figure 4.1). Deletion of the first 30 amino acids of HBxAg rendered the product inactive (Faktor and Shaul, 1990) while removal of 11 or 12 carboxy-terminal residues did not affect the function of HBxAg (Levrero *et al.*, 1990a; Unger and Shaul, 1990). Takada and Koike (1990) cite unpublished observations that deletion of 22 carboxy-terminal residues, removing the carboxy-termimal conserved region, abolished its transactivating capacity. In contrast, Levrero *et al.* (1990a) indicate that HBxAg with 37 residues deleted from its carboxyl-terminus, removing half of the negatively charged region with predicted helical structure, retained the ability to transactivate. However, there are two reports indicating that HBxAg truncated at the same amino acid residue was non-functional (Koike *et al.*, 1989; Caselmann *et al.*, 1990). The conflicting results may have arisen from different targets that were used in the transactivation assays (see figure 4.1). If HBxAg functions by mediating protein-protein interactions within the transcription complex (discussed further in section 6A.4.2) it may require alternative segments for interaction with different factors in the systems analysed above.

The targets for transactivation by HBxAg are listed in table 4.1 along with the cell line in which the assays were carried out. HBV promoter/enhancer complexes have been investigated in several systems including, 1) in the context of the complete HBV genome itself (Colgrove *et al.*, 1989; Koike *et al.*, 1989), 2) in the context of the subgenomic mRNA transcription units (Rossner *et al.*, 1990; Zhou and Yen, 1990), 3) as isolated complexes linked to the CAT gene (Jameel and Siddiqui, 1986; Twu and Schloemer, 1987; Spandau and Lee, 1988; Wollersheim *et al.*, 1988; Siddiqui *et al.*, 1989; Faktor and Shaul, 1990; Levrero *et al.*, 1990a; Unger and Shaul, 1990), and 4) with the firefly luciferase reporter gene inserted into the complete HBV genome (Raney *et al.*, 1990). Removal of the enhancer region from its complex with the core promoter fused to a reporter gene

FIGURE 4.1 Effects of deletions on the transactivating capacity of HBxAg

Top: Representation of the complete amino acid sequence of HBxAg. Conserved (cons.) regions and negatively charged α-helical region (see section 1.3.4) are indicated.

Below: Boxes represent the portions of HBxAg contained in deletion mutants. Transactivation activity (+) or lack of transactivation activity (-) are indicated along with the targets that were assayed.

Abbreviations:

HBV enh/Xp - HBV enhancer and X promoter region fused to the CAT gene.

HBV E site - Three tandem copies of an oligonucleotide representing HBV nucleotides 2459-2484 were inserted 3´ to the CAT gene under control of the tk promoter.

HIV1 or HIV2 LTR- LTR of HIV1 or HIV2 fused to the CAT gene.

HBV genome - the truncated HBxAg polypeptide was created by a frameshift mutation in the X ORF contained within the complete HBV genome. The level of HBV-specific transcripts was assessed in cells transiently transfected with the mutant HBV genome and compared to the level produced by cells transiently tranfected with an intact HBV genome.

References:

a) Faktor and Shaul (1990).

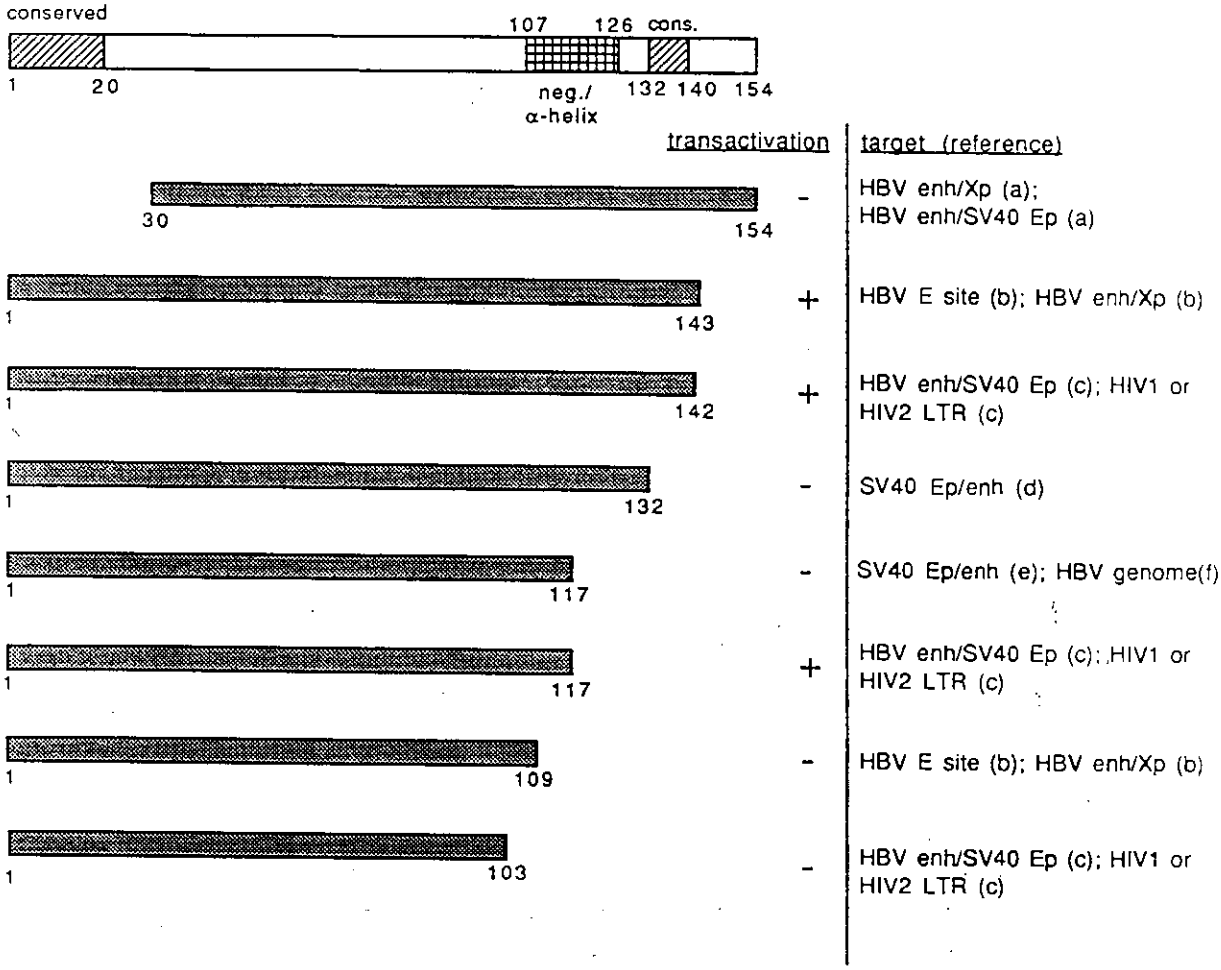
b) Unger and Shaul (1990).

c) Levrero *et al.* (1990a).

d) Takada and Koike (1990).

e) Caselmann *et al.* (1990).

f) Koike *et al.* (1989).


conserved
107
126
cons.
1
20
neg./
α-helix
132
140
154
transactivation
target (reference)
30
154
-
HBV enh/Xp (a);
HBV enh/SV40 Ep (a)
1
143
+
HBV E site (b); HBV enh/Xp (b)
1
142
+
HBV enh/SV40 Ep (c); HIV1 or
HIV2 LTR (c)
1
132
-
SV40 Ep/enh (d)
1
117
-
SV40 Ep/enh (e); HBV genome(f)
1
117
+
HBV enh/SV40 Ep (c); HIV1 or
HIV2 LTR (c)
1
109
-
HBV E site (b); HBV enh/Xp (b)
1
103
-
HBV enh/SV40 Ep (c); HIV1 or
HIV2 LTR (c)

(Spandau and Lee, 1988; Colgrove *et al.*, 1989; Siddiqui *et al.*, 1989) dramatically reduced susceptibility to transactivation by HBxAg indicating that this region serves as a target for transactivation within the HBV genome. Numerous transcriptional regulatory sequences from heterologous viruses have been reported as targets for transactivation by HBxAg. In certain cases (for example the SV40 early promoter/enhancer in Vero cells, or the HIV1 LTR in HepG2 or HeLa cells) conflicting results have been obtained for the capacity of HBxAg to transactivate a certain regulatory sequence in a specific cell line. This may be due to: 1) differences in the basal activity of the reporter alone; a high basal activity could potentially mask transactivation, for example, in the case of the SV40 early promoter/enhancer in Vero cells (Twu and Schloemer, 1987); 2) variations in the HBxAg expressing plasmid construction used; if HBxAg was expressed at a very high level under control of a particular promoter the phenomenon of "squelching" (Ptashne, 1988) could mask transactivation (see section 4.3 for further discusion), and 3) individual variations in the CAT assays.

The most detailed study of cell type specificity for HBxAg transactivation has been carried out by Seto and his colleagues (1989). They concluded that transactivation is cell-line specific. Transactivation was observed in human cells of varying tissue origin and in mouse and monkey cells. Divergent results were obtained for a particular target in cell lines of similar cell-type origin, and only a subset of promoters was transactivated in any particular cell type (for example, the RSV LTR versus the HIV1 LTR in Jurkat, T-lymphoma cells). The implications of these observations on the mechanism of transactivation by HBxAg will be discussed below.

It has been demonstrated that several cellular regulatory sequences are targets for transactivation by HBxAg, and these results may explain certain physiological phenomena associated with HBV infection. Aberrant expression of MHC class II antigen on HBV infected hepatocytes (reviewed by Van den Oord, 1990) may be due to transactivation by HBxAg of the regulatory region of the genes that encode these antigens (Hu *et al.*, 1990). The expression of MHC class II antigens by hepatocytes may have a variety of consequences. Hepatocytes may become antigen presenting cells to T-helper lymphocytes which recognise foreign antigen in association with this class of MHC antigen. T-helper cells secrete lymphokines to activate cytotoxic T-lymphocytes (CTLs), so activation of T-helper cells by an infected hepatocyte could increase hepatocytolysis mediated by CTLs. Antigen presenting hepatocytes may also become susceptible to the direct cytolytic effects of a subset of T-helper cells shown to have these properties (Siliciano *et al.*, 1988).

Conversely, MHC class II expression by infected hepatocytes may function to inactivate T-helper cells by presentation of antigen in the absence of the ability of the presenting cell to secrete lymphokines (reviewed by Meuller *et al.*, 1989), thereby promoting the development of chronic infection.

Transactivation of MHC class I gene expression by HBxAg may contribute to the elevated expression of these antigens on the surface of hepatocytes during HBV infection (Pignatelli *et al.*, 1986). Zhou and his colleagues (1990) have shown that the promoter region of the murine MHC class I gene, $H2K^b$, is a target for transactivation by HBxAg in human cells; this result is consistent with elevated production of MHC class I antigen by HepG2 cells stably transfected with a dimer of the HBV genome and producing virus. Increased MHC class I expression may augment hepatocyte necrosis mediated by CTLs.

The susceptibility of the human β-interferon promoter to transactivation by HBxAg (Twu and Schloemer, 1987) is a curious result as increased serum interferon level is not associated with HBV infection (Hill *et al.*, 1971). This effect of HBxAg may be blocked *in vivo* by repression of the β-interferon promoter by HBcAg (Twu and Schloemer, 1989). Indeed, elevated β-interferon mRNA was not detected in HepG2 cells stably transfected with a dimer of the HBV genome and producing virus (Zhou *et al.*, 1990). Alternatively, these results may highlight the importance of chromatin environment in rendering a particular target promoter susceptible to transactivation. Twu and Schloemer (1987) observed transactivation of an episomal β-interferon promoter while the endogenous gene is not a target for transactivation by HBxAg expressed from an integrated HBV genome (Zhou *et al.*, 1990). A similar effect has been observed for transactivation of the β-globin promoter in episomal configuration by adenovirus E1a protein while this result was not observed for the endogenous promoter (Green *et al.*, 1983).

Increased c-*myc* mRNA has been detected in mouse fibroblasts (NIH3T3) stably transfected with the X ORF under control of the SV40 early promoter/enhancer complex (Koike *et al.*, 1989). In addition, transient assays in the same cell line revealed that the c-*myc* promoters fused to the CAT gene were susceptible to transactivation by HBxAg. An increase in c-*myc* expression could contribute to the development of HCC associated with HBV infection and may have implications for the mechanism of transactivation by HBxAg (see Chapter 6).

A striking serological feature of HBV infection is the presence of a vast quantity of HBsAg. High titre serum may contain 10^{13} particles per ml with HBsAg particles present in vast excess over virion (Cossart, 1971). The HBsAg particles may serve as "decoy

virions" to bind neutralising antibodies produced in response to infection. The work described in this chapter is an investigation of the effect of HBxAg on expression of HBsAg in transfected hepatoma cells. Expression of all three forms of HBsAg polypeptide is considered and preliminary studies on the segment of the HBV genome that serves as a target for transactivation by the *X*-gene product are described.

4.2 Results

4.2.1 Plasmid constructions

Plasmid constructions are diagrammed in figure 4.2. Plasmid pHBV1004 contains the transcription units for the preS2/S mRNAs and the X mRNA and was constructed by Jackson (1987) as follows: HBV sequences (1005-3182/1-88) were isolated from plasmid pHBV130 (Gough and Murray, 1982) by complete digestion with *Bgl*II followed by partial digestion with *Bam*HI. The resulting 2.3 kb fragment was ligated into the *Bam*HI site of pUC8. Plasmid pHBV1004-B contains a frameshift mutation that was introduced within the X-coding sequence in plasmid pHBV1004 by partial digestion with *Bam*HI, filling in cohesive ends of the site at position 2682 with DNA polymerase I (Klenow fragment), and ligation of the resulting blunt ends of plasmid DNA. The presence of four bp inserted at position 2683 was confirmed by DNA sequence determination (figure 4.3). The mutant X ORF contains a frameshift after the triplet coding for amino acid residue 10 (aspartate) in the X coding sequence resulting in the creation of a stop codon after 14 missense codons. To confirm that the preS2/S promoter sequences remained identical in the parent and mutant plasmids, DNA sequence was determined on the fragment between nucleotide positions 1004-1413 contained in the two plasmids. The integrity of the entire X ORF contained in plasmid pHBV1004 was confirmed by DNA sequence determination.

Plasmid pS1B contains the transcription units for the preS1 mRNA, the preS2/S mRNAs, and the frameshift-mutant X mRNA and was constructed as follows: HBV sequences (733-1413) were isolated from plasmid pHBV130 by digestion with *Bcl*I and *Xho*I. Plasmid pHBV1004-B was digested with *Bam*HI and *Xho*I and the resulting 4.6 kb, vector-containing fragment was ligated to the 680 bp fragment isolated from plasmid pHBV130. The mutant X ORF in plasmid pS1B was replaced with the wild-type X ORF by the following series of manipulations to create plasmid pS14. HBV sequences (2655-3182/1-88) were isolated from plasmid pHBV1004 by digestion with *Nco*I and *Hind*III.

FIGURE 4.2 Plasmid constructions

A) Nucleotide position of ORFs in three translational reading frames (a,b,c) in the HBV sequence is indicated. Numbers indicate the first and last coding nucleotide. Transcription control regions are as follows: spI = preS1 promoter, spII = preS2/S promoter, En/Xp = enhancer/X promoter, Cp = core promoter. Filled in arrows represent transcription initiation sites. ▲ indicates HBV polyadenylation signal at nucleotide position 16.

B) HBV sequences contained within plasmid constructions used in this study. Details of construction are described in the text. Restriction endonuclease cleavage sites within the HBV sequence and in the polylinker sequence flanking the HBV DNA insert are indicated. Numbers above restriction sites represent the position of the first nucleotide of the recognition sequence in the HBV genome. "X f.s." indicates the site of the four bp insert creating a frameshift mutation in the X ORF. HBV polyadenylation site is indicated as in "A".

C) HBxAg expression plasmid (pSV2HBX) and corresponding mutant plasmid containing frameshift mutation in the X ORF. Transcription in these plasmids is under control of the SV40 enhancer (*)/ early promoter (▶) complex.

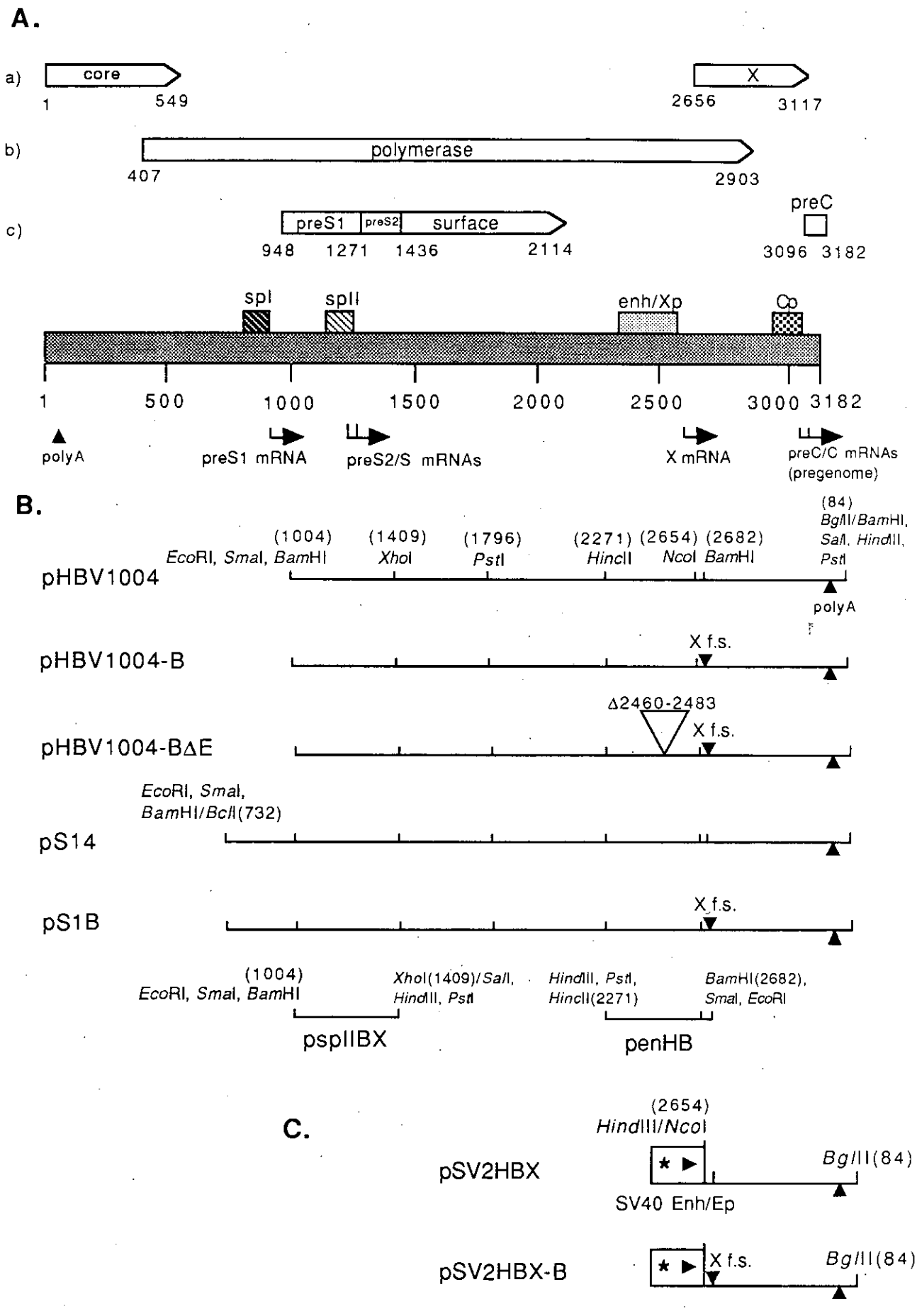
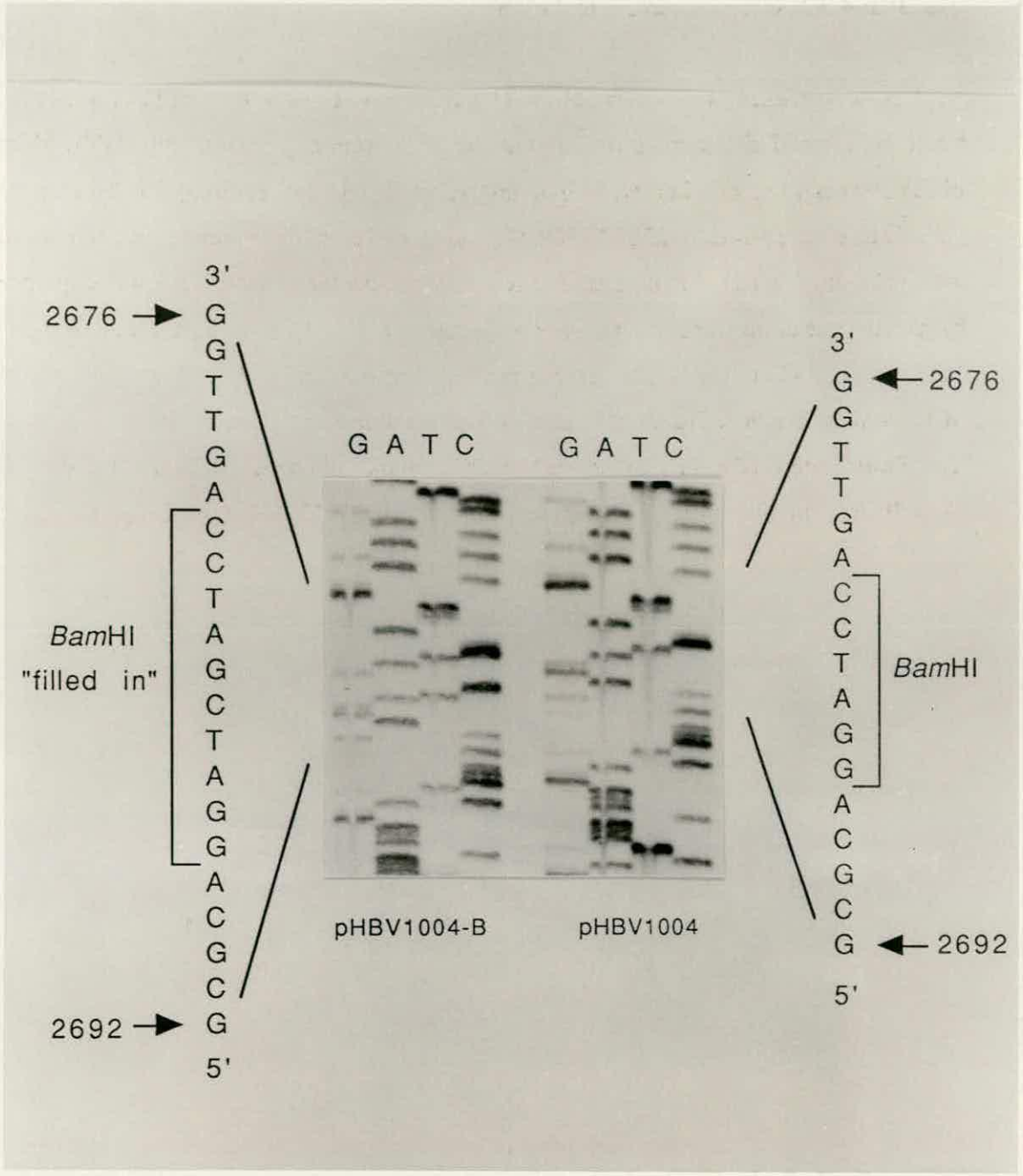

A.
a)
core
1
549
X
2656
3117
b)
polymerase
407
2903
c)
preS1
preS2
surface
preC
948
1271
1436
2114
3096
3182
spI
spII
enh/Xp
Cp
1
500
1000
1500
2000
2500
3000
3182
polyA
preS1 mRNA
preS2/S mRNAs
X mRNA
preC/C mRNAs
(pregenome)
B.
(1004)
EcoRI, SmaI, BamHI
(1409)
XhoI
(1796)
PstI
(2271)
HincII
(2654)
NcoI
(2682)
BamHI
(84)
BglII/BamHI,
SalI, HindIII,
PstI
pHBV1004
polyA
pHBV1004-B
X f.s.
pHBV1004-BΔE
Δ2460-2483
X f.s.
EcoRI, SmaI,
BamHI/BclI(732)
pS14
pS1B
X f.s.
(1004)
EcoRI, SmaI, BamHI
XhoI(1409)/SalI,
HindIII, PstI
HindIII, PstI,
HincII(2271)
BamHI(2682),
SmaI, EcoRI
pspIIBX
penHB
C.
(2654)
HindIII/NcoI
pSV2HBX
BglII(84)
SV40 Enh/Ep
pSV2HBX-B
X f.s.
BglII(84)

FIGURE 4.3 X ORF frameshift mutation

Nucleotide sequence of plasmids pHBV1004 and pHBV1004-B showing insertion of four bp at the *Bam*HI site at position 2682 in the HBV genome. Plasmids pHBV1004 and pHBV1004-B were digested with *Sph*I and *Hind*III and the resulting 768 bp fragment (HBV nucleotide positions 2514-3182/1-88) was ligated to m13mp19 linearised by digestion with *Sph*I and *Hind*III. Single stranded recombinant bacteriophage DNA was sequenced by dideoxynucleotide chain termination (Sanger *et al.*, 1977; section 2B.7) using the M13mp series -20 universal sequencing primer. The sequence of the non-coding strand of HBV was obtained. Numbers represent the nucleotide position in the HBV genome. The *Bam*HI site (HBV nucleotide position 2682) in plasmid pHBV1004 and the "filled-in" *Bam*HI site containing the four bp insert in plasmid pHBV1004-B are indicated.


3'
2676
G
G
T
T
G
A
C
C
T
A
G
C
T
A
G
G
A
C
G
C
G
2692
5'
BamHI
"filled in"
G A T C
G A T C
pHBV1004-B
pHBV1004
3'
G
2676
G
T
T
G
A
C
C
T
A
G
G
A
C
G
C
G
2692
5'
BamHI

Plasmid pS1B was digested with *Nco*I and *Hind*III and the resulting 4.9 kb, vector-containing fragment was ligated to the 615 bp fragment from pHBV1004.

Plasmid pHBV1004-BΔE was constructed by site directed mutagenesis resulting in deletion of HBV nucleotide positions 2460-2483 from plasmid pHBV1004-B (figure 4.4). HBV sequences contained in plasmid pHBV1004-B were isolated by digestion with *Eco*RI and *Hind*III and the resulting 2.3 kb fragment was ligated to the bacteriophage vector m13mp19 linearised by digestion with *Eco*RI and *Hind*III. Single stranded recombinant bacteriophage DNA was purified by "large-scale" preparation (section 2B.2.4) and mutagenesis was carried out as described (section 2B.9).

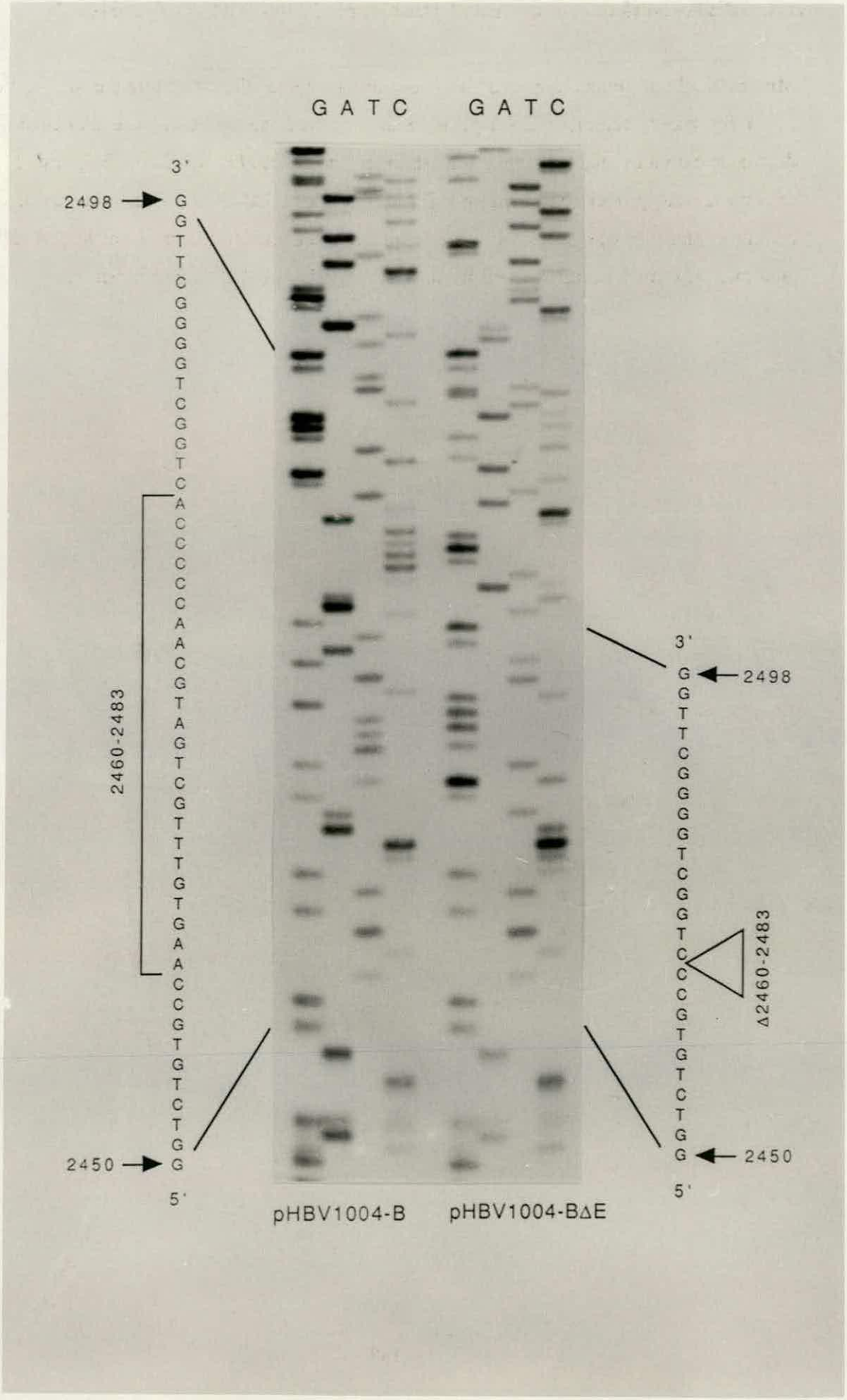
Plasmid pSV2HBX was constructed by H. Houston to express HBxAg. Plasmid pSV2βG (a gift from P. Southern) contains the rabbit β-globin gene under control of the SV40 early promoter/enhancer complex (Gorman *et al.*, 1982). The rabbit β-globin structural sequences were excised by digestion with *Hind*III, filling in cohesive ends with DNA polymerase I (Klenow fragment), and digestion with *Bgl*II. HBV sequences (2655-3182/1-88) were isolated from plasmid pHBV130 by digestion with *Nco*I, filling in cohesive ends with T4 DNA polymerase and digestion with *Bgl*II. The resulting 615 bp fragment was ligated to the 4.2 kb vector fragment of pSV2βG. The integrity of the X ORF in plasmid pSV2HBX was confirmed by DNA sequence determination. Plasmid pSV2HBX-B contains the same frameshift mutation in the *X*-gene as pHBV1004-B and was constructed by an equivalent series of reactions and confirmed by DNA sequence determination.

Plasmid pspIIBX contains the preS2/S promoter of HBV and was constructed as follows: HBV sequences (1004-1413) were isolated from plasmid pHBV1004 by digestion with *Eco*RI and *Xho*I and the resulting 409 bp fragment was ligated to plasmid pUC8 linearised by digestion with *Eco*RI and *Sal*I. Plasmid penHB contains the enhancer/X promoter region of HBV and was constructed as follows: HBV sequences (2274-2686) were isolated from plasmid pHBV1004 by digestion with *Hinc*II and *Bam*HI and the resulting 412 bp fragment was ligated to plasmid pUC8 linearised by digestion with *Hinc*II and *Bam*HI.

Plasmids containing the human growth hormone (HGH) gene under control of the mouse metallothionine promoter or the herpes simplex tk promoter were obtained from Nichols Institute (San Juan Capistrano, California). The plasmid containing the HGH gene under control of the murine hypoxanthine ribosyl transferase (HPRT) promoter was constructed as follows: Plasmid pDWMΔ322 (a gift from D. Melton) contains the HPRT

FIGURE 4.4 Nucleotide sequence of plasmids pHBV1004 and pHBV1004-BΔE

Mutant single stranded template DNA obtained by site directed mutagenesis (section 2B.9) and parent template DNA prior to site directed mutagenesis were sequenced by dideoxynucleotide chain termination (Sanger *et al.*, 1977; section 2B.7) using an oligonucleotide primer representing HBV nucleotides 2383-2399. The sequence of the coding strand was obtained. Numbers represent the nucleotide position in the HBV genome. The nucleotides deleted from plasmid pHBV1004-BΔE are indicated.


G A T C
G A T C
3'
2498
G
G T T C G G G G G T C G G T C A C C C C C A A C G T A G T C G T T T G T G A A C C G T G T C T G G
2450
5'
2460-2483
3'
G
2498
G T T C G G G G G T C G G T C C C G T G T C T G G
2450
5'
Δ2460-2483
pHBV1004-B
pHBV1004-BΔE

promoter (-845 to +40, relative to the transcription initiation site) inserted into plasmid pUC8. The HPRT promoter (-637 to +40) was isolated by digestion with *Bam*HI and *Bgl*II and was ligated into the *Bam*HI site of plasmid p0GH containing the HGH coding sequence.

Plasmid p1243 was a gift of H. Meade and contains the human serum albumin (HSA) gene under control of the caseine promoter. All plasmid DNA was prepared from cells of *E. coli* strain ED8654 harbouring the plasmid as described in section 2B.2.4 ("large-scale" preparation).

4.2.2 Transient expression of HBsAg

In order to investigate the effect of HBxAg on surface antigen production, hepatoma cells (HepG2 or HuH7) were transiently transfected with both genes contained in a single plasmid construction (pHBV1004) which maintains the native configuration of the genes and their complete transcription units including all transcriptional regulatory elements. Differentiated hepatoma cell lines permissive for HBV replication (Sureau *et al.*, 1986; Yaginuma *et al.*, 1987b) were used to match the host and tissue tropism demonstrated by HBV during natural infection. The cell-lines used were devoid of integrated HBV sequences in order to eliminate interference in the assay by viral antigens produced from integrated sequences. Hepatoma cells transfected with pHBV1004 secreted HBsAg into the culture medium where it was detected 4-5 days post-transfection by radio-immunoassay (AUSRIA II-125, Abbott Laboratories). This sensitive assay for the viral antigen eliminated the need for a heterologous reporter. Indeed, the HBsAg coding sequence itself has been used as a reporter for the activity of heterologous promoters (Marschall *et al.*, 1989).

Supercoiled plasmid DNA was used for transient transfections, as Jackson (1987) had shown that linearisation of the plasmid with a restriction endonuclease that detached the preS2/S promoter from the plasmid vector sequences did not affect HBsAg production upon transient transfection of mouse L cells. This indicated that the plasmid vector sequences did not contain a cryptic eukaryotic promoter that could direct transcription of the surface mRNAs. Transient transfection was used in order to maintain the episomal nature of input DNA thus mimicking the state of HBV DNA in the hepatocyte during acute infection. The transfection procedure employed was a modification of the DEAE-dextran/chloroquine method described by Luthman and Magnusson (1983) and was

developed by Jackson (1987). The chloroquine enhancement procedure was compared to other enhancing treatments (Lopata *et al.*, 1984) and shown to be the most effective for both HepG2 and HUH7 cells (table 4.2).

Table 4.2 Assessment of enhancement protocols on transfection efficiency

	P/N Values	
	HepG2	**HuH7**
DEAE-dextran alone[a]	4.7	17.9
DEAE-dextran + chloroquine[b]	26.5	25.6
DEAE-dextran + glycerol[c]	7.1	18.2
DEAE-dextran + DMSO[d]	9.3	19.2

Radio-immunoassay (AUSRIA II-125) was carried out on medium recovered from cells transfected with 30 μg of pHBV1004 per plate.
[a] Transfection protocol was as described in section 2B.1.2 except chloroquine was not added to the transfection solution.
[b] Transfection protocol was as described in section 2B.1.2
[c] DEAE-dextran/DNA mixture was as described in section 2B.1.2. After incubation for 8 hours, transfection mixture was removed and cells were incubated in 4 ml of 10% (v/v) DMSO in HBS [25mM Hepes.KOH, 0.27mM $Na_2HPO_4.2H_2O$, 140mM NaCl, pH 7.1] for 4 minutes at room temperature. The DMSO solution was removed, and cells were washed and incubated as described.
[d] As for "c" but the enhancing solution was 15% (v/v) glycerol in HBS.

4.2.3 Frameshift mutation in the X gene reduces transient expression of HBsAg

Transfection of hepatoma cells with plasmid pHBV1004-B, containing a frameshift mutation in the X coding sequence, resulted in significant reduction in HBsAg synthesis compared to cells transfected with an equivalent amount of pHBV1004 (figure 4.5). The difference in HBsAg expression between the two plasmids was greatest when small amounts of DNA were used for transfection; HBsAg secretion by HepG2 cells transfected with 3 μg of pHBV1004 was an average of fivefold (and as much as sevenfold) higher than by cells transfected with 3 μg of pHBV1004-B, but with 10 μg or 30 μg of the plasmids the difference was two to threefold in this series of experiments. With larger amounts of DNA the availability of transcription factors that are not influenced by HBxAg may become limiting. Although it is possible it is unlikely that the stability of the transcript encoding HBsAg was affected by the 4 bp insertion in the X coding sequence, for it has

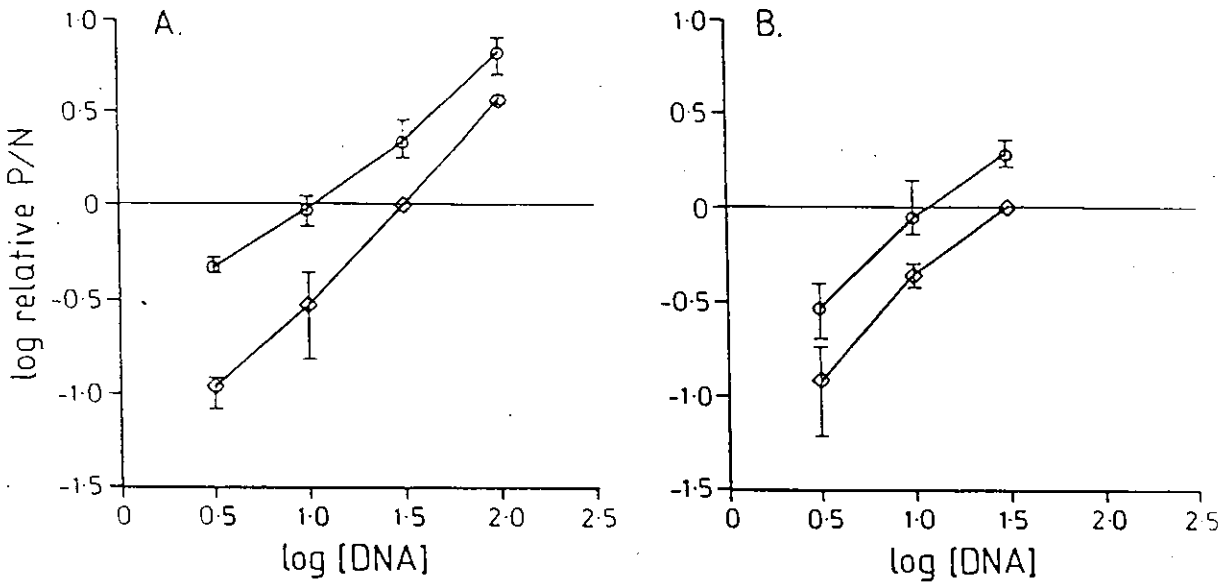


FIGURE 4.5 Transient expression of HBxAg

Levels of HBsAg production from cells transfected with HBsAg expression plasmids pHBV1004 or pHBV1004-B. HBsAg was detected by radio-immunoassay (AUSRIA II125) of either the cell culture medium or medium that had been concentrated 30-fold to bring the values within the linear range of the assay. The level of HBsAg expression was initially calculated as a ratio of counts per minute obtained for the sample to counts per minute obtained from the medium of mock transfected cells (P/N). The P/N values within each experiment were then normalised to the P/N value obtained for cells transfected with 30 μg of pHBV1004-B (relative P/N value). This allows comparison of the values obtained in different experiments in which the absolute level of HBsAg expression may differ. The average relative P/N values were calculated by weighting the relative P/N values in each experiment according to the number of plates used for transfection with each amount of DNA. Error bars indicate the range of average values obtained in each experiment; A) HepG2 cells; B) HuH7 cells; (○), cells transfected with pHBV1004; (◇) cells transfected with pHBV1004-B.

been shown that even a 270 bp deletion of the HBV enhancer contained within this mRNA did not affect its stability in COS cells (Bulla and Siddiqui, 1988).

Attempts to find an internal control against which to compare absolute levels of HBsAg produced by cells transfected with pHBV1004 or pHBV1004-B proved unsuccessful. Human growth hormone (HGH) is a secreted product, and it was believed that this reporter may provide a useful internal control, as the medium could be assayed in parallel for HBsAg and HGH by radio-immunoassays (AUSRIA II-125, and Allegro supplied by Nichols Institute, respectively). HuH7 cells were transiently transfected with plasmids containing the HGH gene under transcriptional control of the mouse metallothionine, the herpes simplex virus tk, or the murine HPRT promoter in the presence (cotransfection with pSV2HBX) or absence (cotransfection with pSV2HBX-B) of HBxAg. A slight increase in HGH expression (1.2-1.5 fold, data not shown) was observed from all three plasmids in the presence of HBxAg. While these values were low, they were not suitable as internal controls. It had been reported that the SV40 early promoter devoid of the 72 bp repeat enhancer was not a target for transactivation by HBxAg (Siddiqui *et al.*, 1989), however, the emergence of conflicting reports (Koike *et al.*, 1989) eliminated the suitability of this promoter in the internal control plasmid, and it was not used. Comparison of the absolute levels of HBsAg secretion (as reflected in P/N values) should be a reliable indication of the ability of each plasmid to direct expression of HBsAg. The transfection efficiencies achieved with the parent and mutant plasmids should be identical as they only differ in 4 bp inserted in pHBV1004-B, and the DEAE-dextran transfection procedure used in transient protocols is more reproducible than other procedures (Ausubel *et al.*, 1989). Plasmid DNAs were prepared in parallel, and several different preparations of the two plasmids were used throughout the course of these experiments.

4.2.4 Detection of different forms of HBsAg

Plasmid pHBV1004-B contains the transcription units for both middle- and major-S polypeptides. The proportion of surface antigen particles on which middle-S (preS2 containing) polypeptides could be detected was determined by radio-immunoassay in which the solid phase was a polystyrene bead coated with anti-preS2 monoclonal antibody, Q19/10 (Heerman *et al.*, 1988), and the bound antigen was detected with [^{125}I]-labelled human anti-HBsAg serum provided with the AUSRIA II-125 HBsAg detection kit. Table

4.3 shows that transfection of hepatoma cells with pHBV1004 resulted in the secretion of a greater number of HBsAg particles containing detectable preS2 epitopes than transfection with an equivalent amount of pHBV1004-B. The proportion of secreted HBsAg particles containing detectable preS2 epitopes was similar with both parent and mutant plasmids and these ratios were similar to that obtained with serum derived HBsAg particles.

Table 4.3 PreS2 epitopes on HBsAg preparations

	Expt. I[a]		Expt. II		Expt. III	
source of HBsAg	S[b]	preS2	S	preS2	S	preS2
serum[c]	24.8	7.5	23.5	11.11	23.9	8.9
yeast[d]	N.T.	N.T.	25.9	1.2	23.2	1.1
pHBV1004[e]	16.8	5.7	39.2	26.2	20.5	5.8
pHBV1004-B	5.8	1.8	18.7	7.5	6.4	1.4

[a]P/N values are shown from three independent experiments.
[b]HBsAg was detected with the AUSRIA II-125 kit. (Detection of preS2 is described in the text).
[c]HBsAg derived from serum (20 ng/ml) was provided with the AUSRIA II-125 kit as a positive control.
[d]HBsAg derived from yeast was purified from *Saccharomyces cerevisiae* harbouring plasmid pHING2 (Murray *et al.*, 1984), which contains the major-S polypeptide coding region under control of the yeast *PHO5* promoter.
[e]pHBV1004 and pHBV1004-B preparations are 30-fold concentrated medium from HepG2 cells transfected with 30 μg of the indicated plasmid.
N.T. = not tested.

Two procedures were employed in an effort to directly observe the different forms of HBsAg produced in transient assays. 1) HBsAg concentrated from cell culture medium by ultracentrifugation and extracts of cells transfected with pHBV1004 or pHBV1004-B were fractionated by SDS-PAGE and analysed by immunoblotting. 2) Cells transiently transfected with pHBV1004 or pHBV1004-B were metabolically labelled with [^{35}S]-methionine and [^{35}S]-cysteine, and HBsAg particles in the media and whole cell extracts were immunoprecipitated and fractionated by SDS-PAGE. Neither procedure revealed any HBsAg specific band in comparison with mock-transfected cells, most likely due to the low amount of HBsAg present. Thus on the assumption that the proportion of middle-S polypeptides on each HBsAg particle was the same for those expressed from either the parent or mutant plasmid, transfection of cells with the mutant plasmid yielded reduced

expression of middle-S polypeptides.

4.2.5 Complementation of the mutation in the *X*-gene

The frameshift mutation in the X coding sequence contained in pHBV1004-B could be complemented by supplying HBxAg in *trans*. In the experiments summarised in figure 4.6, hepatoma cells were transfected with 30 μg of pHBV1004-B. This gave a level of HBsAg expression that could be readily assayed and that was about half that from the parent plasmid, pHBV1004. Cotransfection of HepG2 cells with pHBV1004-B and a plasmid carrying the X coding sequence under control of the SV40 early promoter/enhancer complex (pSV2HBX) restored HBsAg production to a level similar to that produced by cells transfected with an equivalent amount of pHBV1004 (figure 4.6a). HBxAg was supplied in *trans* under control of a heterologous promoter instead of its own promoter/enhancer complex to avoid competition for transcription factors specifically interacting with the HBV enhancer contained in the HBsAg expression plasmids which may be important for transcription from the preS2/S promoter (see below).

Cotransfection can give rise to a concatanated network of input DNA, and thus it was possible that the observed effect was due to a *cis* effect of the SV40 transcriptional control signals contained in pSV2HBX. As a control, hepatoma cells were cotransfected with pHBV1004-B and pSV2HBX-B, which contained the same frameshift mutation in the X coding sequence. HBsAg expression in HepG2 cells was then equivalent to that of cells transfected with pHBV1004-B alone (figure 4.6a). This established that the restoration of HBsAg expression to wild-type levels by cotransfection with the plasmid pSV2HBX was due to a *trans* effect of HBxAg.

In HuH7 cells, HBsAg production from plasmid pHBV1004-B was increased only 1.5 fold upon cotransfection with 30 μg of pSV2HBX and was not completely restored to the level produced by cells transfected with pHBV1004. Transactivation of HBsAg expression by HBxAg in HuH7 cells displayed concentration dependence; with higher amounts of HBxAg, transactivation was reduced or no longer observed (table 4.4). Therefore, the 30μg of pSV2HBX used in the experiment summarised in figure 4.6a may have been greater than the amount needed for optimal transactivation. Concentration dependence for transactivation of HBsAg expression was not observed in HepG2 cells; a linear increase in HBsAg expression from plasmid pHBV1004-B was observed upon cotransfection with increasing amounts of pSV2HBX up to 100 μg (table 4.4).

FIGURE 4.6

A) HBsAg production. Levels of HBsAg production from cells transfected with HBsAg expression plasmids or cotransfected with HBsAg plasmid and HBxAg expression plasmid. Calculation of the Relative P/N Value is described in the legend to figure 4.5. Except where indicated, the values presented here are an average from at least three experiments (* indicates values that are an average from two experiments). Error bars indicate the range of average values obtained in each experiment. Key to plasmids used for transfection: 4 = pHBV1004; B = pHBV1004-B; X = pSV2HBX; XB = pSV2HBX-B; "μg of DNA" is the amount of DNA used to transfect each plate of cells.

B) Northern blot of RNA from cells expressing HBsAg. Total cytoplasmic RNA was prepared (section 2B.2.3) from three plates of cells, four days post-transfection with A) 30 μg pUC8 per plate; B) 30 μg pHBV1004; C) 30 μg pHBV1004-B; D) 30 μg pHBV1004-B plus 30 μg pSV2HBX; E) 30 μg pHBV1004-B plus 30 μg pSV2HBX-B. The surface transcript probe was a 795 bp *Bam*HI/*Pst*I fragment from pHBV1004 representing HBV nucleotide positions 1005-1800, and film was exposed to the membrane for three days. The probe for HSA transcripts was a 1.8 kb *Bam*HI fragment from plasmid p1243, and exposure was 30 minutes. Densitometric analysis of the exposed X-ray film was done on the Shimadzu Dual Wavelength Cromato Scanner Model CS-930. The numbers under the curves represent the area under each peak.

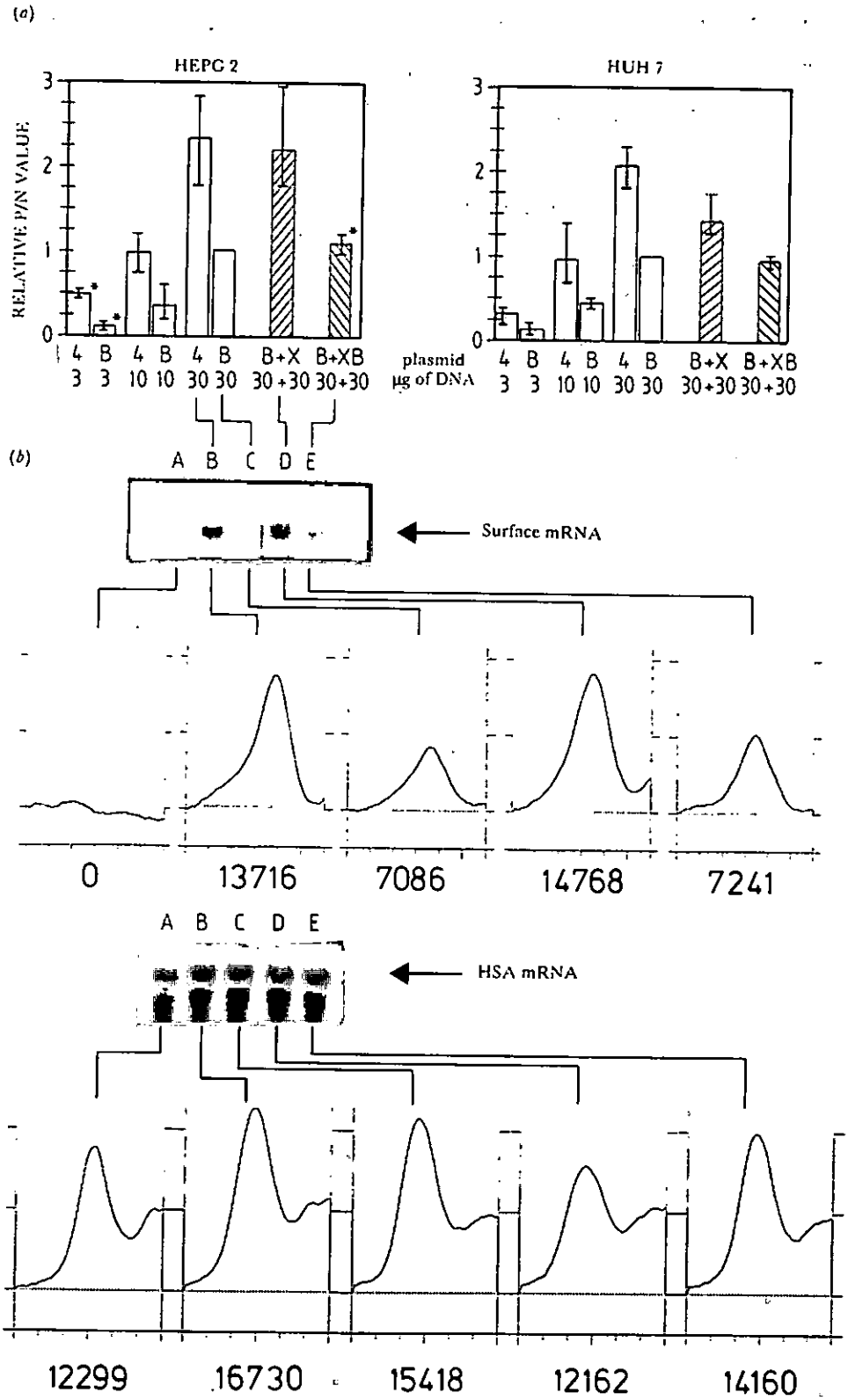

(a)
HEPG 2
HUH 7
RELATIVE P/N VALUE
plasmid
µg of DNA
4 B 4 B 4 B B+X B+XB
3 3 10 10 30 30 30+30 30+30
(b)
A B C D E
Surface mRNA
0
13716
7086
14768
7241
HSA mRNA
12299
16730
15418
12162
14160

Table 4.4 Concentration dependence of transactivation by HBxAg in HuH7 cells

	P/N Values	
Plasmid(s) used for transfection	**HepG2**	**HuH7**
pHBV1004 (30 μg)	7.5	18.4
pHBV1004-B (30 μg)	3.2	7.9
pHBV1004-B(30μg); pSV2HBX(10μg)	6.3	12.9
pHBV1004-B(30μg); pSV2HBX(30μg)	9.6	10.1
pHBV1004-B(30μg); pSV2HBX(100μg)	22.1	6.3

Radio-immunoassay (AUSRIA II-125) was carried out on medium recovered from cells transfected with the indicated amounts of the indicated plasmid(s) per plate.

4.2.6 HBxAg modulates the level of preS2/S mRNAs

Total cytoplasmic RNA extracted from cells transfected with HBsAg expression plasmids and from cells cotransfected with pHBV1004-B and the HBxAg expression plasmid or its corresponding frameshift mutant was fractionated by agarose gel electrophoresis and transferred to a nylon membrane. Hybridisation to a [^{32}P]-labelled DNA probe containing sequences from the preS and surface ORFs revealed that the level of ~2kb preS2/S mRNAs corresponded to the amount of HBsAg produced (figure 4.6b). This probe was stripped from the membrane, which was then rehybridised with a [^{32}P]-labelled DNA fragment from the plasmid p1243, a probe for HSA transcripts. The densitometer traces in the lower part of figure 4.6b showed that the samples analysed contained equivalent amounts of RNA; all the values lie within -13% to +18% of the mean, the reproducibility of individual scans of the various bands being $\pm$7%.

4.2.7 Detection of an X-specific transcript

A nylon membrane containing fractionated RNAs identical to those described above was hybridised to a [^{32}P]-labelled DNA probe (nucleotide positions 2683-3182/1-88) containing sequences complementary to the 3´ end of the preS2/S mRNAs and to the X mRNA. Only the transcripts that had been detected with the preS/surface-specific probe were detected with this probe (data not shown). No transcripts were detected from the

X-gene under control of its own promoter (pHBV1004, pHBV1004-B) or a heterologous promoter (pSV2HBX, pSV2HBX-B). Some investigators have detected a ~1kb transcript by northern blot analysis in human hepatoma cells transiently transfected with HBxAg under control of its own promoter in the context of the transcription unit encoding HBsAg (Siddiqui *et al.*, 1986; Bulla and Siddiqui, 1988, 1989; Koike *et al.*, 1989) or under control of a heterologous promoter (Siddiqui *et al.*, 1987; Spandau and Lee, 1988; Zahm *et al.*, 1988; Koike *et al.*, 1989). Others have not detected an X-specific transcript by northern blot analysis even in hepatoma cells stably transfected with the X coding sequence under control of its own promoter and have resorted to amplification by the polymerase chain reaction (PCR) to detect the X transcript (Aufiero and Schneider, 1990). PCR could not be used to detect the X transcript in cells transfected with pHBV1004 or pHBV1004-B, as it is completely contained within the preS2/S mRNAs and could not be distinguished with oligonucleotide primers for PCR. PCR was carried out using as a template total cellular RNA from HepG2 cells transfected with pSV2HBX or pSV2HBX-B. An X-specific band was amplified in both cases (figure 4.7) indicating that a very low level of transcription is taking place in these cells directed by the SV40 early promoter/enhancer complex.

Two transcripts have been detected in hepatoma cells transiently transfected with a plasmid construction similar to pSV2HBX which contains the SV40 polyadenylation site ~800 bp downstream of the HBV polyadenylation site (Spandau and Lee, 1988; Koike *et al.*, 1989). These two forms presumably arise from heterologous termination of transcription. The PCR primers used in this study would not distinguish between the two possible forms of transcript.

An *X*-gene product has been detected in several transient systems in which X mRNA is transcribed under control of its own promoter (Chang *et al.*, 1987) or a heterologous promoter (Siddiqui *et al.*, 1987; Levrero *et al.*, 1990b). Three procedures were employed unsuccessfully to detect the *X*-gene product expressed by plasmid pHBV1004 or pSV2HBX. 1) Extracts of transfected cells were fractionated by SDS-PAGE and analysed by immunoblotting with anti-HBxAg serum. 2) Transfected cells were permeabilised and incubated with anti-HBxAg serum and then with secondary antibody conjugated to fluorescein isothiocyanate (FITC) and analysed by fluorescence microscopy. 3) Transfected cells were permeabilised in suspension (Schroff *et al.*, 1984), incubated with anti-sera as above and analysed by fluorescence activated cell sorting.

FIGURE 4.7 Amplification of low abundance mRNAs by PCR

Total cellular RNA was prepared (see section 2B.2.3) from two or three plates of HepG2 cells, four days post-transfection with 100 μg per plate of the indicated plasmid DNA except in the case of pHBV1004 in which 30 μg DNA per plate was used. "Mock" indicates cells subjected to the transfection procedure in the absence of input DNA. PCR was carried out on samples treated with DNaseI or with DNase I and RNase A as described (section 2B.8). PCR products were fractionated by size by agarose gel electrophoresis, transferred to a nylon membrane and detected by hybridisation to a [^{32}P]-labelled DNA probe representing sequences internal to both oligonucleotide primers.

A) Detection of X mRNA. Oligonucleotide primers used were SV40 nucleotides 5228-5206 and HBV antisense strand nucleotides 3182-3163 yielding an amplified DNA fragment of 590 bp. The probe for amplified X transcripts was a 323 bp *Bam*HI/*Dra*I fragment from pHBV1004 representing HBV nucleotide positions 2683-3006. Key to plasmids used for transfection: X = pSV2HBX; XB = pSV2HBX-B. Film was exposed to the membrane for 48 hours.

B) Detection of preS1 mRNA. Oligonucleotide primers used were HBV sense strand, nucleotides 914-937 with eight additional nucleotides at the 5′ end including a cleavage site for *Pst*I; and HBV antisense strand, nucleotides 1236-1217 with eight additional nucleotides at the 5′ end including a cleavage site for *Eco*RI. The amplified DNA fragment was 338 bp long. The probe for amplified preS1 transcripts was a 145 bp *Eco*RI/*Ban*II fragment from pHBV1004 representing HBV nucleotide positions 1004-1149. Key to plasmids used for transfection: 1004 = pHBV1004; S14 = pS14; S1B = pS1B. Film was exposed to the membrane for 16 hours.

A.
kb
1.0
0.5
0.3
mock, DNase
X, DNase
X, DNase/RNAse
XB, DNase
XB, DNase/RNAse
B.
1004, DNase
S14, DNase
S14, DNase/RNase
S1B, DNase
S1B, DNase/RNase

4.2.8 HBxAg activates expression of the large-S polypeptide

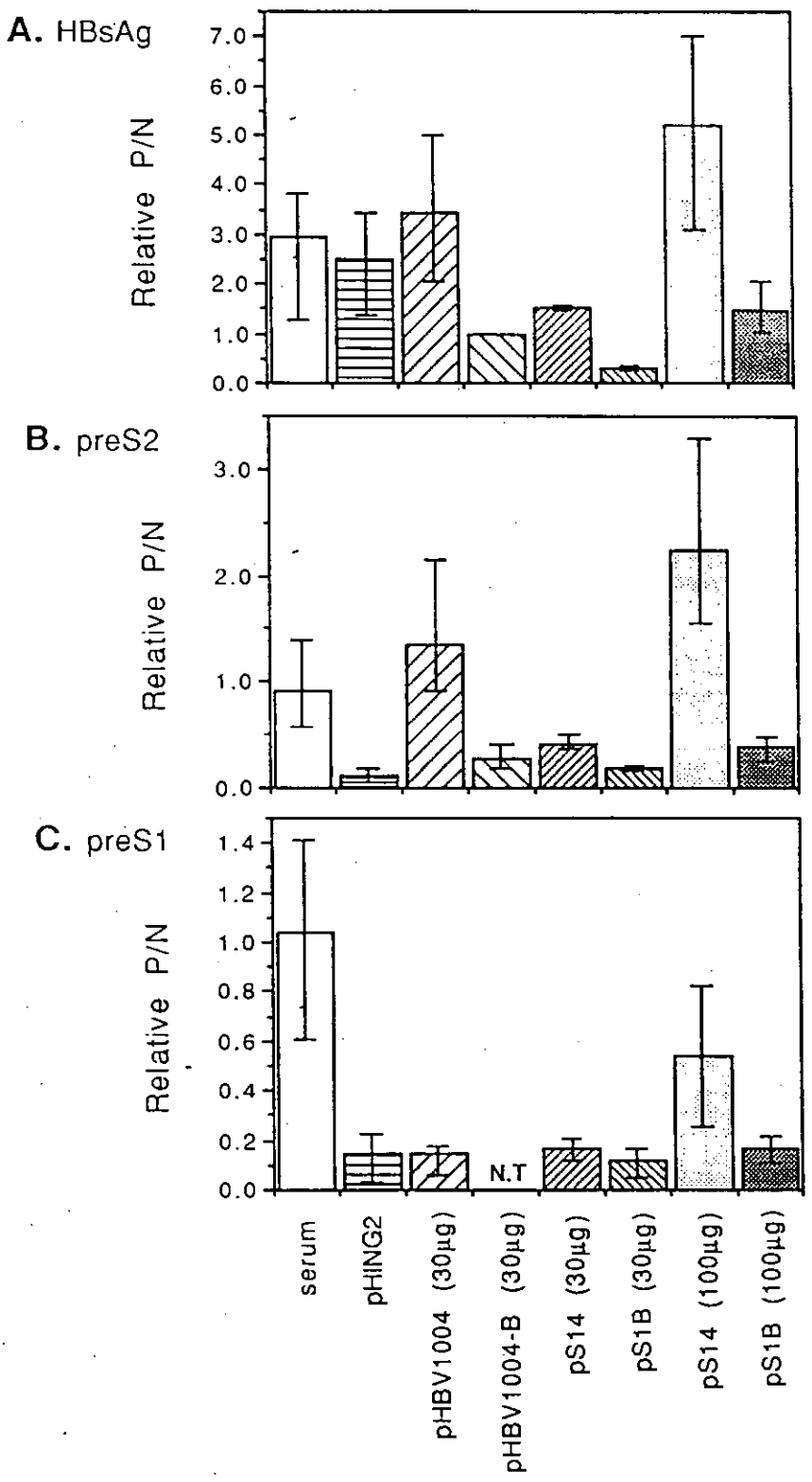
Plasmid pHBV1004 contained only the preS2/S promoter and not the preS1 promoter located ~300 bp upstream which directs synthesis of the 2.3 kb subgenomic mRNA encoding the large, preS1/preS2/surface polypeptide. These two promoters in isolation fused to a reporter gene have similar transcriptional activities in differentiated hepatoma cells (Siddiqui *et al.*, 1986; Antonucci and Rutter, 1989; Bulla and Siddiqui, 1989; Chang and Ting, 1989). However the preS2/S promoter displays much higher activity in several systems including HBV infected liver (Cattaneo *et al.*, 1984), the HCC cell-line, PLC/PRF/5 (Ou and Rutter, 1985), hepatoma cells transiently (Chang *et al.*, 1987; Yaginuma *et al.*, 1987b) or stably (Sureau *et al.*, 1986) transfected with the complete HBV genome and producing virus, and hepatoma cells transiently tranfected with a plasmid contruction containing a reporter gene fused to the respective promoters in the context of the complete viral genome (Raney *et al.*, 1990). There is a precedent for a single viral polypeptide acting as both repressor and transactivator. The SV40 large T-antigen represses transcription from the viral early promoter and activates the late transcription program (reviewed by Khoury *et al.*, 1987). Bulla and Siddiqui (1989) described a negative regulatory region within the preS1 coding region that, when removed, greatly increased transcription from the preS1 promoter; all of the plasmid constructions used in their study expressed X mRNA, and it remained to be investigated whether HBxAg played a role in downward modulation of transcription from the preS1 promoter.

Plasmid pS14 contains the complete transcription unit for the mRNAs encoding HBxAg and all three forms of HBsAg. Plasmid pS1B contains the same frameshift mutation in the *X* gene as pHBV1004-B. These plasmids were used to transfect HepG2 cells and the proportion of secreted surface antigen particles containing detectable large-S polypeptides was determined by radio-immunoassay in which the solid phase was a polystyrene bead coated with anti-preS1 monoclonal antibody, MA18/7 (Heerman *et al.*, 1984). PreS2 epitopes and total HBsAg were detected as described above.

Transfection of cells with plasmid pS14 or pS1B led to approximately threefold reduction in the total amount of HBsAg secreted compared to an equivalent amount of the corresponding preS2/S expressing plasmid (figure 4.8a; compare pS14 (30 μg) vs. pHBV1004 (30 μg) and pS1B (30 μg) vs. pHBV1004-B (30 μg)). A corresponding reduction was also observed in the number of secreted HBsAg particles on which preS2 epitopes were detected (figure 4.8b). It was possible that expression of the large-S

FIGURE 4.8 PreS epitopes on HBsAg preparation

HepG2 cells were transfected with the indicated amount of the various plasmids and radio-immunoassays were carried out four days post-transfection on cell culture medium concentrated 30-fold by ultracentrifugation. HBsAg derived from serum (20 ng/ml) was provided with the AUSRIA II-125 kit as a positive control. HBsAg derived from yeast was purified from *Saccharomyces cerevisiae* harbouring plasmid pHING2 (Murray *et al.*, 1984), which contains the major-S polypeptide coding region under control of the yeast *PHO5* promoter. A)HBsAg was detected with the AUSRIA II-125 kit. B,C) PreS2 and preS1 epitopes were detected as described in the text (sections 4.2.4 and 4.2.8, respectively). Relative P/N values were calculated as described in the legend to figure 4.5. Note the differences in scale in the three histograms. N.T. = not tested.


A. HBsAg
Relative P/N
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
B. preS2
Relative P/N
3.0
2.0
1.0
0.0
C. preS1
Relative P/N
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.T
serum
pHING2
pHBV1004 (30μg)
pHBV1004-B (30μg)
pS14 (30μg)
pS1B (30μg)
pS14 (100μg)
pS1B (100μg)

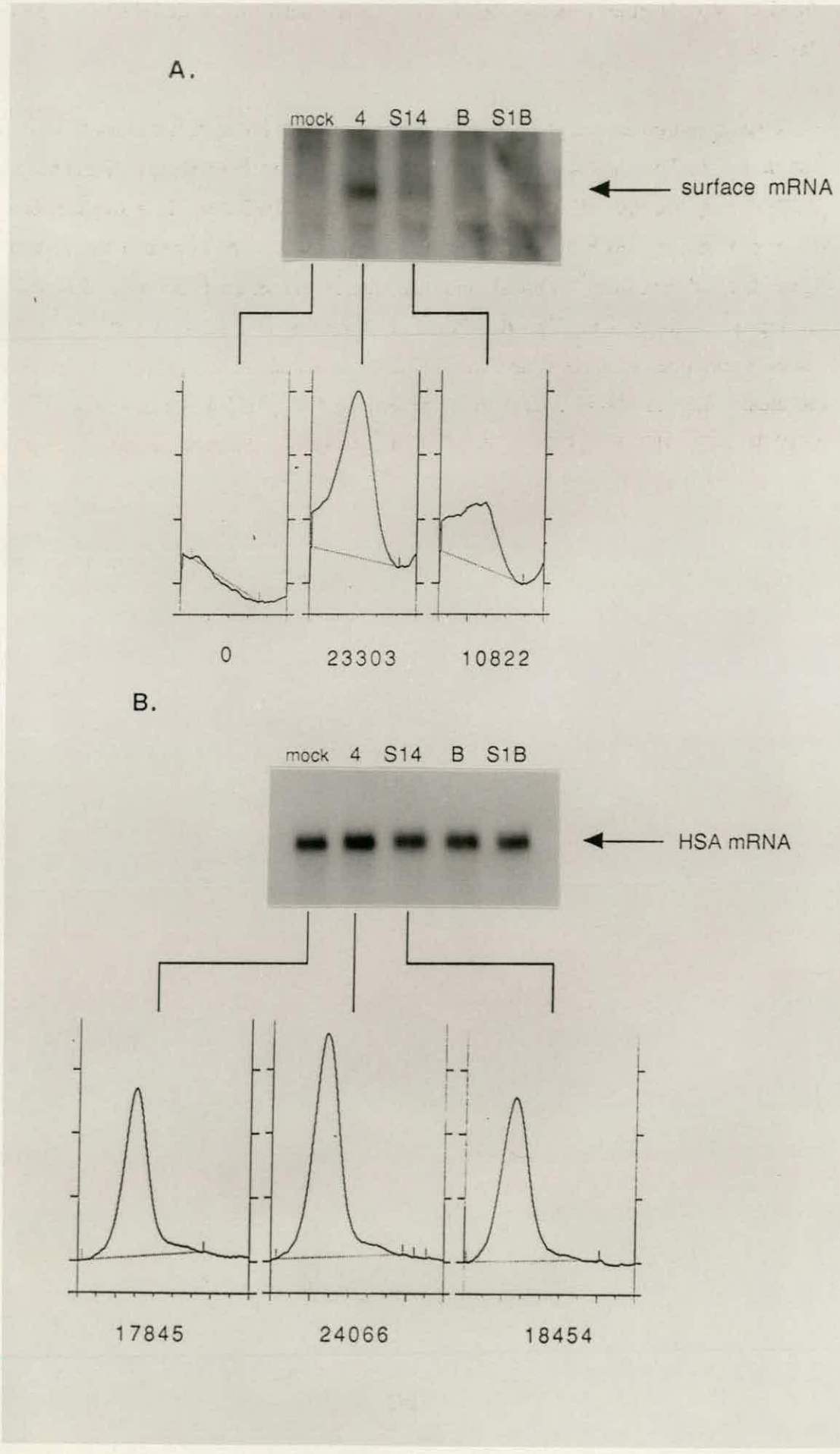
polypeptides was inhibiting secretion of HBsAg particles as has been observed by several investigators in systems where the large-S polypeptide is overexpressed under control of a heterologous promoter (Laub *et al.*, 1983; Chisari *et al.*, 1986; Persing *et al.*, 1986; McLachlan *et al.*, 1987; Ou and Rutter, 1987; Molnar-Kimber *et al.*, 1988). Soluble and insoluble proteins extracted by freeze-thaw lysis of transfected cells were subjected to radio-immunoassay in an attempt to quantify intracellular HBsAg. Very low amounts of HBsAg were detected (barely above the threshold for positive vs. negative determination) and no conclusions could be drawn from these experiments (data not shown). Procedures described above (section 4.2.4) for detection of different forms of HBsAg in transfected cells proved unsuccessful.

Total cellular RNA was extracted from cells transfected with pS14 or pS1B or an equivalent amount of the corresponding preS2/S expressing plasmid. RNA was fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridised to a [^{32}P]-labelled DNA probe complementary to the surface and X mRNAs. Transfection of cells with pS14 led to a reduction in the level of preS2/S mRNAs compared to that in cells transfected with an equivalent amount of pHBV1004 corresponding to the reduction in HBsAg secreted (figure 4.9). As above, no X-specific mRNA was detected by this analysis. The 2.3 kb mRNA encoding the large-S polypeptide was not abundant enough to detect by this method but could be detected by amplification using PCR (figure 4.7). The low level of preS1 containing mRNA compared to preS2/S mRNAs that was observed in this system is consistent with that observed in other systems described above.

Transfection of hepatoma cells with pS14 resulted in secretion of a greater number of HBsAg particles on which preS1 polypeptides could be detected compared to tranfection with pS1B (figure 4.8c). Therefore, HBxAg does not repress transcription from the preS1 promoter, but appears to transactivate it. The proportion of HBsAg particles on which preS1 epitopes could be detected was lower in the transient expression system used in this study than that in human serum supplied as a positive control with the HBsAg detection kit. This could be due to the presence of 22 nm filaments in the human serum which contain a higher proportion of preS1 containing polypeptides than 22 nm particles (Heerman *et al.*, 1984). Analysis by electron microscopy of particulate forms secreted by cells transfected with HBsAg expression plasmids proved unsuccessful. Previous studies have revealed that transient or stable transfection of mouse or monkey cells with the transcription unit for the preS1 mRNA or with the complete HBV genome

FIGURE 4.9 Northern blot of RNA from cells transfected with HBsAg expression plasmids

RNA was prepared from 2 plates of cells transfected with 30 μg of the indicated plasmid per plate. A) The surface transcript probe was a 587 bp *Bam*HI/*Bgl*II fragment from pHBV130 representing HBV nucleotide positions 2683-3182/1-88. Film was exposed to the membrane for seven days. B) The HSA probe was as described in the legend to figure 4.6, and exposure was for 30 minutes. Densitometer analysis was as described in the legend to figure 4.6. The densitometer traces in "B" indicated that the samples analysed contained equivalent amounts of RNA; all values lie within -11% to +20% of the mean. Key to plasmids used for transfection: 4 = pHBV1004; S14 = pS14; B = pHBV1004-B; S1B = pS1B. "Mock" indicates cells subjected to the transfection procedure in the absence of input DNA.


A.
mock
4
S14
B
S1B
surface mRNA
0
23303
10822
B.
mock
4
S14
B
S1B
HSA mRNA
17845
24066
18454

yielded secretion of only 22nm HBsAg particles and not filamentous structures (Dubois *et al.*, 1980; Stratowa *et al.*, 1982; Siddiqui, 1983; Stenlund *et al.*, 1983; Wang *et al.*, 1983; Zelent *et al.*, 1987). However, stable or transient transfection of human hepatoma cells with the complete HBV genome resulted in secretion of filamentous forms of HBsAg (Sureau *et al.*, 1986; Chang *et al.*, 1987; Sells *et al.*, 1987). No reports could be found concerning HBsAg forms secreted by human hepatoma cells transiently transfected with a subgenomic fragment of HBV containing the transcription unit encoding large-S polypeptides. The lack of filaments produced in mouse and monkey cells could be due to the preference of the preS1 promoter for differentiated hepatoma cells (Chang and Ting, 1989; Raney *et al.*, 1990; see section 1.4.2).

4.2.9 Investigation of the HBxAg response element of HBV

Shaul and Ben-Levy (1987) described several regions within the HBV enhancer sequence that bound nuclear proteins extracted from differentiated hepatoma cells. When individual factor-binding regions were isolated and fused in multiple repeat downstream of the β-globin transcription unit, one region (2459-2484, designated "E") conferred susceptibility to transactivation by HBxAg (Faktor and Shaul, 1990). This region was deleted from plasmid pHBV1004-B by site directed mutagenesis to create plasmid pHBV1004-BΔE. The effect of this deletion on HBsAg expression was investigated in the X-mutant plasmid in order to eliminate as a factor any effect that the deletion might have on expression of HBxAg itself. Transient transfection of HuH7 cells with plasmid pHBV1004-BΔE resulted in nearly fourfold reduction in HBsAg production compared to transfection with pHBV1004-B (table 4.5a). This effect was not observed in HepG2 cells. These results were obtained on several occasions when the two cell lines were transfected on the same day with the same plasmid DNA preparations. An anomaly of the results obtained with HepG2 cells in this series of experiments is that the amount of HBsAg produced by cells transfected with pHBV1004-B was lower than that observed with pHBV1004 to a greater extent than had been seen previously as described in section 4.2.3 (1:6.2 compared to 1:2.2). This change in expression profile was not observed in HuH7 cells and continued to be observed in HepG2 cells recovered from the original stock culture indicating that the result was not due to a change in the cells due to passaging.

The ability of HBxAg to modulate expression of HBsAg from the E-deletion plasmid construction was investigated (table 4.5b). Cotransfection of HepG2 cells with

Table 4.5a Affect of E-deletion on secretion of HBsAg in hepatoma cells.

	Average P/N Values ± Standard Deviation	
Plasmid(s) used for transfection:	**HepG2**	**HuH7**
pHBV1004 (30 μg)	22.0 ± 9.9	39.3 ± 10.1
pHBV1004-B (30 μg)	3.5 ± 2.1	16.1 ± 7.3
pHBV1004-BΔE (30 μg)	3.0 ± 1.4	4.1 ± 2.3

Radio-immunoassay (AUSRIA II-125) was carried out on 30-fold concentrated medium from cells transfected with the indicated amount of the indicated plasmid per plate. Average P/N values were calculated by weighting the P/N values obtained in each experiment according to the number of plates used for tranfection with each plasmid.

Table 4.5b Effect of E-deletion on susceptibility to transactivation by HBxAg

	P/N Value	
Plasmid(s) used for transfection:	**Expt. I**	**Expt. II**
pHBV1004 (30 μg)	29.1	25.0
pHBV1004-B (30 μg)	5.9	2.4
pHBV1004-BΔE (30 μg)	4.7	2.4
pHBV1004-B(30μg); pSV2HBX(30μg)	7.5	4.0
pHBV1004-B(30μg); pSV2HBX-B(30μg)	4.9	3.1
pHBV1004-BΔE(30μg); pSV2HBX(30μg)	6.9	2.5
pHBV1004-BΔE(30μg); pSV2HBX-B(30μg)	3.9	1.8
pHBV1004-B(30μg); pSV2HBX(100μg)	12.5	4.5
pHBV1004-B(30μg); pSV2HBX-B(100μg)	4.9	3.0
pHBV1004-BΔE(30μg); pSV2HBX(100μg)	8.2	3.9
pHBV1004-BΔE(30μg); pSV2HBX-B(100μg)	4.0	1.9

Radio-immunoassay (AUSRIA II-125) was carried out on 30-fold concentrated medium from HepG2 cells transfected with the indicated amount of the indicated plasmid per plate. Results from two independent experiments are shown.

pHBV1004-BΔE and pSV2HBX increased HBsAg expression in the same proportion as cotransfection of cells with its parent plasmid (pHBV1004-B) and the HBxAg expression plasmid. These results, however, were anomalous with those described in section 4.2.5, in that HBsAg production by HepG2 cells cotransfected with plasmid pHBV1004-B and pSV2HBX was not restored to the level observed from cells transfected with plasmid

pHBV1004 although a greater than twofold increase was observed. Similar results were obtained with preparations of pSV2HBX from *E. coli* transformed with plasmid DNA from the original stock indicating that this result was not due to a spontaneous mutation in the HBxAg coding sequence in plasmid pSV2HBX. The similar response in HBsAg production from plasmids pHBV1004-B and pHBV1004-BΔE to HBxAg supplied in *trans* suggests that other regions of the HBV genome contained in these plasmids are capable of mediating transactivation by HBxAg.

In order to examine a larger region of the HBV genome for an HBxAg response element(s), template competition experiments were carried out *in vivo*. The transcriptional control regions for HBsAg contained in plasmid pHBV1004 are the preS2/S promoter (HBV nucleotide positions 1069-1267; Raney *et al.*, 1989) and the enhancer (HBV nucleotide positions 2350-2550; section 1.4.3a). These regions were subcloned into plasmid pUC8 generating pspIIBX (HBV nucleotide positions 1004-1413) and penHB (HBV nucleotide positions 2271-2686).

Template competition experiments were carried out by cotransfection of HuH7 cells with pHBV1004-B and pSV2HBX in the presence of increasing concentrations of competitor plasmid. Competitor DNA should reduce the transactivating potential of HBxAg on HBsAg expression if HBxAg binds directly to sequences contained in the competitor plasmid or if the effect of HBxAg is mediated by cellular transcription factors present in limiting concentration that bind to those sequences. In the absence of the influence of the *X*-gene product both penHB and pspIIBX could compete for factors necessary for transcription from the preS2/S promoter (figure 4.10, "pHBV1004-B (10μg)"). However, when pSV2HBX was cotransfected with pHBV1004 and either competitor template, expression of HBsAg was increased by an equivalent amount to that in which no competitor was present (figure 4.10 a,b). This suggested that the effect of HBxAg could be mediated by sequences outwith the regions contained in the competitor template plasmids.

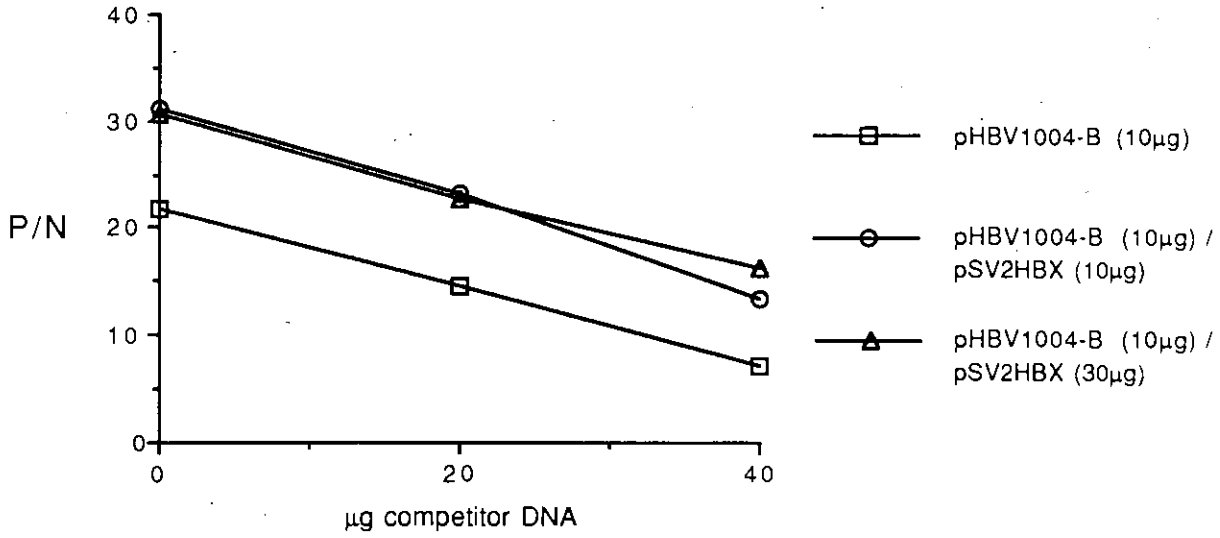
4.3 Discussion

The results presented in this chapter demonstrate that HBxAg can function to increase transient expression of HBsAg in two different human hepatoma cell lines transfected with a plasmid (pHBV1004) encoding the preS2/S mRNAs. A frameshift mutation close to the 5′ end of the X coding sequence in the HBsAg expression vector

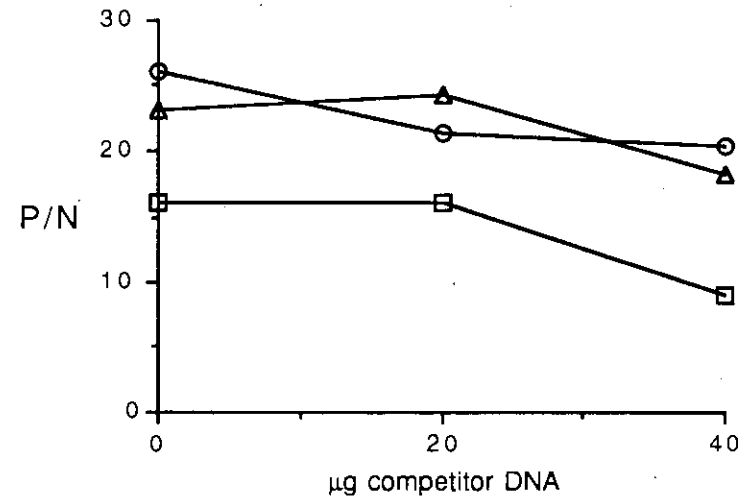
FIGURE 4.10 *In vivo* competition for transacting factors

HuH7 cells were transfected with HBsAg expression plasmid, pHBV1004-B, in the absence (0 μg competitor DNA) or presence of increasing amounts of competitor plasmid DNA (□). μg of DNA indicated in the figure refers to the amount of plasmid DNA per plate used for transfection. Competition for transactivation by HBxAg was assayed by cotransfection of pHBV1004-B with 10 μg (○) or 30 μg (△) pSV2HBX in the absence (0 μg competitor DNA) or presence of increasing amounts of competitor plasmid DNA. Competitor plasmids were approximately half the size of pHBV1004-B and were added in four-fold (20 μg competitor DNA) or eight-fold (40 μg competitor DNA) molar excess relative to pHBV1004-B. Competitor plasmids contained: A) The HBV enhancer region (nucleotide positions 2271-2686), penHB; or B) the preS2/S promoter (nucleotide positions 1004-1413), pspIIBX. C) The vector sequence contained in the two competitor plasmids (pUC8) was used as a negative control. Each point in "A" or "C" is the average P/N value obtained from two independent experiments; the actual values ranged from $\pm$1% to $\pm$26 percent of the mean. Each point in "B" is the actual P/N value obtained from a single experiment.

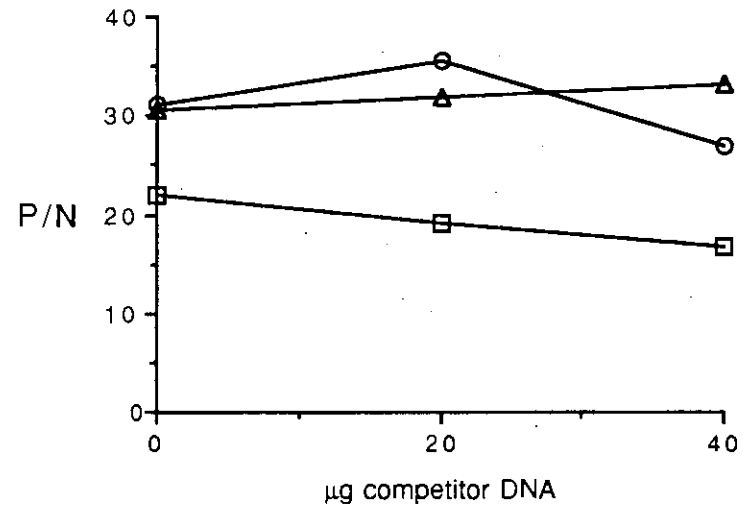
A. penHB



B. pspIIBX



C. pUC



significantly reduced the amount of HBsAg secreted into the culture medium. The mutation did not affect the ratio of detectable preS2 epitopes to total HBsAg secreted indicating that HBxAg does not affect those features of HBsAg regulation controlling this ratio including variable initiation of transcription under control of the preS2/S promoter and variable use of initiation codons in the preS2/S mRNA. The mutation in the X ORF in plasmid pHBV1004-B could be complemented by supplying HBxAg in *trans*, which is definitive evidence that HBxAg can modulate expression of another viral gene product.

The levels of HBsAg expression from pHBV1004 or from its corresponding X-frameshift mutant in the absence or presence of HBxAg supplied in *trans* corresponded to the amount of preS2/S mRNA indicating that the HBxAg does not exert its effect at the level of translation or secretion. This effect of HBxAg on steady state levels of mRNA under control of a target promoter has been observed in several systems (Seto *et al.*, 1988; Zahm *et al.*, 1988; Colgrove *et al.*, 1989; Koike *et al.*, 1989; Twu and Robinson 1989; Twu *et al.*, 1989a; Aufiero and Schneider, 1990; Faktor and Shaul, 1990; Hu *et al.*, 1990; Twu *et al.*, 1990; Unger and Shaul, 1990). Analysis of transcription from preformed complexes extracted from nuclei has indicated that HBxAg functions at the level of transcription initiation and not by increasing mRNA stability (Colgrove *et al.*, 1989; Siddiqui *et al.*, 1989; Aufiero and Schneider, 1990; Hu *et al.*, 1990; Levrero *et al.*, 1990a).

Transfection of HepG2 cells with a plasmid containing the transcription units for the preS1 and preS2/S subgenomic mRNAs (pS14) resulted in secretion of HBsAg on which preS1 epitopes could be detected. The total amount of HBsAg secreted and the number of detectable preS1 epitopes were reduced by a similar proportion by a frameshift mutation in the X ORF in this plasmid, indicating that HBxAg is not responsible for the repression of transcription from the preS1 promoter, and suggesting that indeed, HBxAg transactivates transcription from the preS1 promoter to a similar extent to that from the preS2/S promoter.

The total amount of HBsAg secreted by cells transfected with pS14 or pS1B was less than that produced by cells transfected with an equivalent amount of corresponding plasmid encoding only the middle- and major-S polypeptides (pHBV1004 or pHBV1004-B). This corresponded to a reduction in preS2/S mRNAs indicating that the reduction in HBsAg secretion was not due to an inhibition of secretion by the low level of large-S polypeptides produced. It is possible that the effect observed was due to reduced transfection efficiency of the slightly larger preS1 expressing plasmids. Another possible explanation is "promoter occlusion" whereby the activity of a promoter is occluded by

transcription through the promoter region under control of an upstream promoter. This effect would be unexpected due to the very low level of transcription from the upstream, preS1 promoter. However, there are conflicting reports as to the affect of transcription from an upstream promoter on internal initiation of transcription under control of the preS2/S promoter. Secretion of HBsAg was equivalent in COS cells transfected with a plasmid containing only the preS2/S promoter or one containing both the preS2/S and the preS1 promoter (Siddiqui *et al.*, 1986). Although no analysis of mRNA levels was carried out in this study, production of HBsAg particles that contain predominantly major-S polypeptide reflects internal initiation of transcription as middle- and major-S polypeptides are not translated from the preS1 mRNA (Standring *et al.*, 1986). Mouse L cells transfected with a vector containing both native HBsAg promoters produced as much preS2/S mRNA as cells in which transcription of the preS1 mRNA was under control of the strong RSV LTR (Persing *et al.*, 1986). Further, McLachlan and his colleagues (1987) observed middle- and major-S polypeptides produced by NIH3T3 cells stably transfected with a plasmid construction in which the preS1 mRNA was transcribed under control of a retroviral LTR indicating internal initiation even in the presence of a strong upstream promoter, although no comparison was made of the levels of middle- and major-S produced relative to cells transfected with a plasmid encoding the preS2/S mRNA under control of the native promoter. In contrast to the studies mentioned, stable transfection of Chinese hamster ovary cells with a plasmid construction expressing the preS1 mRNA under control of the SV40 early promoter/enhancer resulted in production of only large-S polypeptides indicating a block to internal initiation from the preS2/S promoter (Ou and Rutter, 1987). An experiment that would help elucidate the cause of decreased HBsAg secretion observed in this study from cells transfected with the preS1 expression plasmids would be mutagenesis of the preS1 promoter to ablate its use. Possible targets for mutagenesis are the binding sites for transcription factors Oct-1 and HNF-1 located upstream of the preS1 promoter "TATA-box" (Zhou and Yen, 1991). To definitively rule out inhibition of secretion of HBsAg particles by the large-S polypeptide, a frameshift mutation could be introduced in the preS1 coding region of plasmids pS14 and pS1B.

There have been conflicting reports concerning the ability of HBxAg to transactivate the surface antigen promoters. The data were not shown in support of statements that the surface promoters are not targets for transactivation by HBxAg (Twu *et al.*, 1989a; Siddiqui *et al.*, 1989). These investigations employed target promoters fused to the CAT reporter gene, and it is likely that the results observed were for the isolated surface

antigen promoters in this configuration. Zhou and Yen (1990) have recently reported that transcription from either surface antigen promoter contained within the complete preS1 mRNA transcription unit is not effected by a frameshift mutation following codon 29 of the X ORF. In contrast, there are several reports indicating that the surface antigen promoters are modulated by HBxAg. The luciferase reporter gene was inserted into the complete HBV genome downstream of the preS1 promoter and up to threefold reduction in luciferase activity was obtained upon deletion of the start codon of the X ORF (Raney *et al.*, 1990). Using the HBV mRNAs themselves as a reporter, Colgrove *et al.* (1989) showed that a mutation in the *X*-gene contained within the complete HBV genome led to a reduction in the levels of both the pregenome RNA and the preS2/S mRNA upon transient transfection of HuH7 cells. These results were corroborated by Koike *et al.* (1989) who noted a reduction in pregenome RNA, preS1, preS2/S, and X mRNAs upon transient expression of an X-mutant HBV genome in HepG2 cells. However, in contrast to the results of Colgrove *et al.* (1989), Koike and his colleagues did not see a similar result in HuH7 cells.

The report by Koike *et al.* (1989) highlights several studies noting differences in transcriptional regulatory properties in HepG2 and HuH7 cells. The HBV enhancer II (nucleotide position 2916-3084) placed downstream of the CAT gene under control of the preS1 promoter displayed relatively high enhancer activity in HepG2 cells compared to HuH7 (Yuh and Ting, 1990). Activation of the HIV1 LTR fused to the CAT gene by cotransfection with a plasmid construction expressing HBxAg under control of its own promoter/enhancer complex was significant in HepG2 cells but was not observed in HuH7 cells (Twu and Robinson, 1989).

The results presented in this chapter are consistent with those of Colgrove *et al.* (1989); HuH7 cells were capable of supporting transactivation by HBxAg expressed in its native configuration from within the transcription unit encoding HBsAg. However, in HuH7 cells complementation of the mutation in the *X*-gene in pHBV1004-B was dependent on the concentration of cotransfected plasmid expressing HBxAg, while this effect was not observed in HepG2 cells. The reduction in transactivating potential with increasing concentration of HBxAg expressing plasmid has been observed in other systems (Seto *et al.*, 1990), including in HepG2 cells with respect to transactivation of the HBV X promoter/enhancer complex fused to the CAT gene (Faktor and Shaul, 1990).

Concentration dependence of transactivation has been observed for other transactivating proteins including the yeast Gal4 protein, which turns on the genes

required for galactose metabolism, and the herpes simplex virus VP16 protein, which activates viral early gene transcription (reviewed by Ptashne, 1988). A model proposed to explain this phenomenon (termed "squelching") postulates that transactivators that display this property function by direct protein-protein interactions with cellular transcription factors which are in limiting concentration in the cell (Ptashne, 1988; figure 4.11). Transactivation takes place if these interactions occur in the proper context on the DNA directed by a sequence specific DNA binding site on the activator protein itself or on another protein in the complex. Squelching occurs by overproduction of an activator with respect to target sites which sequesters a limiting cellular transcription factor off of the DNA.

A hypothesis postulating that transactivation is mediated by interaction between HBxAg and one or more cellular factors could explain the ability to transactivate a particular target sequence in one cell line but not in another. If the required cellular factor is present at only a low level or not at all in a particular cell line, it will not support transactivation. A variation in the concentration of a specific factor could explain why squelching was observed in HuH7 cells but not in HepG2 cells. Conflicting observations of transactivation or "squelching" of similar activator and target systems in a particular cell line are more difficult to reconcile but could be due to differences in concentration of transcription factors in particular sublines. The case of concentration-dependent transactivaiton observed in HepG2 cells (Faktor and Shaul, 1990) may have arisen from better transfection efficiency than was obtained in this study or from higher transcriptional activity from the RSV LTR controlling transcription of HBxAg as opposed to the SV40 early promoter/enhancer used in this study.

The region of the HBV genome that is responsible for responding to transactivation by HBxAg was investigated and resulted in observations about the effect of the HBV enhancer on transcription from the preS2/S promoter. Template competition experiments were carried out in an attempt to sequester factors present in limiting concentration (including possibly HBxAg itself) that may be responsible for mediating transactivation of transcription from the preS2/S promoter contained in plasmid pHBV1004-B. Competing templates used contained the preS2/S promoter itself, which displays binding sites for cellular proteins (Raney *et al.*, 1989), or the HBV enhancer region, which serves as a target for transactivation by HBxAg in association with the HBV core promoter or X promoter linked to the CAT gene. (Spandau and Lee, 1988; Wollersheim *et al.*, 1988; Colgrove *et al.*, 1989; Siddiqui *et al.*, 1989). In addition, the HBV enhancer activates

FIGURE 4.11 A model for concentration dependence of transactivation

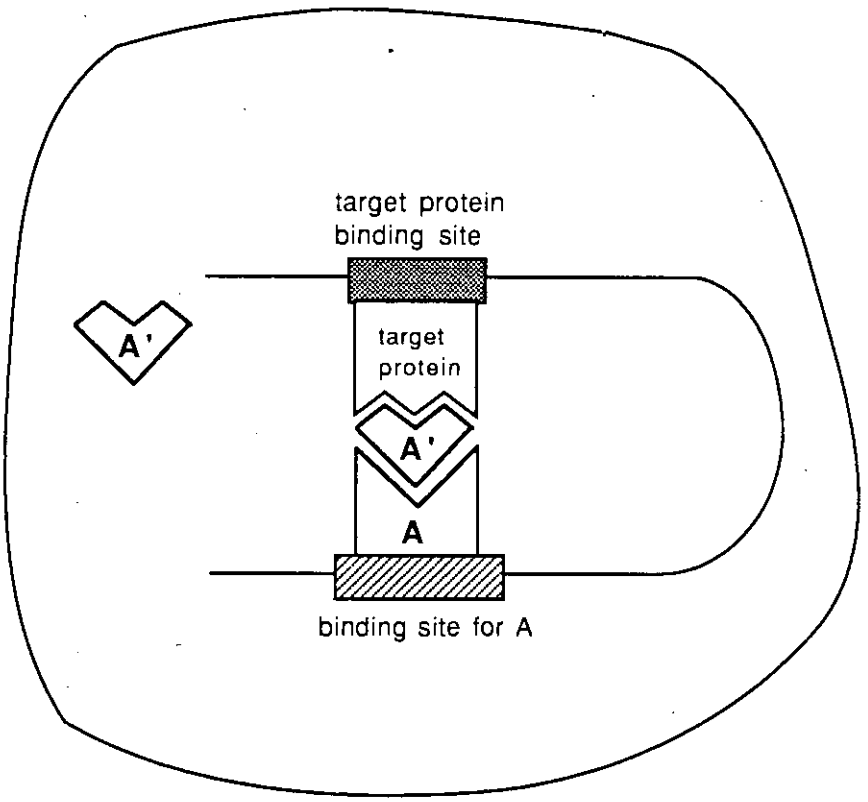
The diagram depicts an activator protein, A´, which does not bind DNA by itself but as a complex with sequence specific DNA binding protein, A.

A) Protein A´ in low concentration mediates interaction of cellular transcription factor, A, with the cellular transcription machinery, "target protein", in the proper context on the DNA.

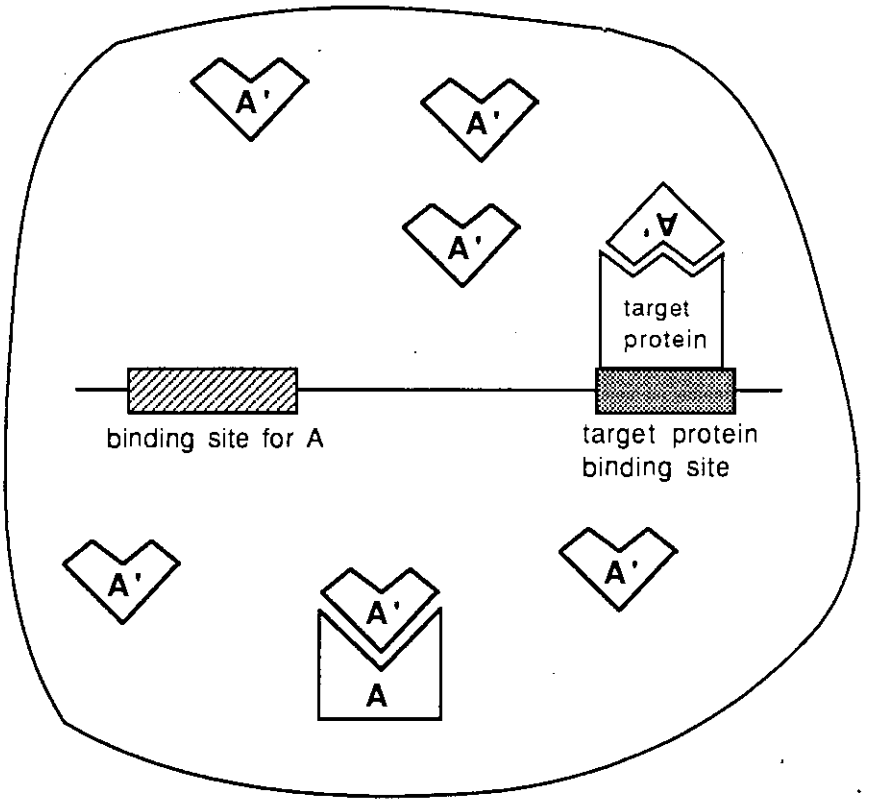
B) Squelching. Overproduction of activator protein A´ sequesters the cellular transcription factor that is in limiting concentration off of the DNA.

Adapted from Ptashne, 1988.

A.


target protein
binding site
A'
target
protein
A'
A
binding site for A

B.


A'
A'
A'
A'
target
protein
binding site for A
target protein
binding site
A'
A'
A'
A

transcription from the preS2/S promoter linked to the CAT gene (Siddiqui *et al.*, 1986; De-Medina *et al.*, 1988; Faktor *et al.*, 1988; Antonucci and Rutter, 1989; Bulla and Siddiqui, 1989; Chang and Ting, 1989; Raney *et al.*, 1989) and increases transient expression of HBsAg from this promoter in hepatoma cells (Bulla and Siddiqui, 1988; Faktor *et al.*, 1988). Cotransfection of competing templates in the absence of HBxAg resulted in a decrease in secretion of HBsAg indicating that cellular *trans*-acting factors interacting with the preS2/S promoter and those that interact with the enhancer region are required for optimal activity of the promoter. These results are consistent with those of Karpen *et al.* (1988) who showed that the HBV enhancer sequence could compete for factors necessary for transcription of the CAT gene under control of the HBV enhancer/core promoter region. In contrast, Pei and Shih (1990) observed that a smaller region of the HBV enhancer (2321-2514, removing the X promoter) cotransfected with the complete HBV genome increased transcription of the pregenome RNA and subgenomic mRNAs. The authors provide evidence that this is due to repression of HBV transcription by the transcription factor CCAAT/enhancer binding protein (C/EBP), however, this also contradicts results obtained in this study (see below).

C/EBP is a heat-stable protein purified from rat liver nuclei (Johnson *et al.*, 1987) that forms a dimer through interaction of repeated leucine motifs ("leucine zipper") and binds DNA in a sequence specific manner (Landschulz *et al.*, 1989). C/EBP binding sites were initially identified as CCAAT motifs and enhancer core motifs (TGTGG(T/A)(T/A)(T/A)G) found in the transcriptional control regions of several viruses (Graves *et al.*, 1986; Johnson *et al.*, 1987). C/EBP displays binding site promiscuity and is capable of activating cellular genes whose product is found specifically in liver cells, for example, albumin (Friedman *et al.*, 1989) and adipose cells (Christy *et al.*, 1989) in which C/EBP is abundant (Birkenmeier *et al.*, 1989).

Purified, recombinant, rat-liver C/EBP binds to the HBV enhancer at at least two sites including the E site described above and a site upstream at nucleotides 2312-2328 (Landschulz *et al.*, 1988a). At increased concentrations C/EBP binds additional sites immediately upstream of the E site (Dikstein *et al.*, 1990b). The E site is highly conserved among all mammalian hepadnaviruses (figure 4.12), although this may be due to functional constraints on the coding sequence of the polymerase ORF contained in this segment. The E site contains the sequence GTGTTTG which is homologous to the SV40 enhancer "core" sequence originally defined as a C/EBP binding site (Johnson *et al.*, 1987). Analysis of native and mutant E sites by DNase I footprinting or by alteration of oligonucleotide

```
           2451                                                    2500
            |                                                       |
HBVadyw    GTCTGTGCCA AGTGTTTGCT GATGCAACCC CCACTGGCTG GGGCTTGGTC
HBVayw     GTCTGTGCCA AGTGTTTGCT GACGCAACCC CCACTGGCTG GGGCTTGGTC
HBVadw     GTCTGTGCCA AGTGTTTGCT GACGCAACCC CCACTGGCTG GGGCTTAGCC
HBVadw2    GTCTGTGCCA AGTGTTTGCT GACGCAACCC CCACTGGCTG GGGCTTGGCC
HBVadr     GTCTCTGCCA AGTGTTTGCT GACGCAACCC CCACTGGATG GGGCTTGGCC
HBVadr4    GTCTCTGCCA AGTGTTTGCT GACGCAACCC CCACTGGATG GGGCTTGGCT
HBVayr     GTCTCTGCCA AGTGTTTGCT GACGCAACCC CCACGGGTTG GGGCTTGGCC
GSHV       TGGTGTGCTC TGTGTTTGCT GACGCAACTC CCACTGGTTG GGGCATTTGC
WHV        TGGTGTGCTC TGTGTTTGCT GACGCAACCC CCACTGGCTG GGGCATTGCC
Consensus  ---T-TGC-- -GTGTTTGCT GA-GCAAC-C CCAC-GG-TG GGGC-T--

                                 E-binding site
```

Figure 4.12: Comparison of E-binding site sequence of HBV clone used in this study (HBV*adyw*) to other mammalian hepadnavirus sequences. Numbers represent nucleotide positions in the sequence of HBV*adyw* (Appendix I). The consensus sequence and the position of the E-binding site defined by Shaul and Ben-Levy (1987) are indicated. Sequence comparison was carried out using the UWGCG "Gap" program and was displayed using the "Pretty" program (Devereux *et al.*, 1984). (References: HBV*adyw*, Pugh *et al.*, 1986; HBV*ayw*, Galibert *et al.*, 1979; HBV*adw*, Ono *et al.*, 1983; HBV*adw*2, Valenzuela *et al.*, 1980; HBV*adr*, Ono *et al.*, 1983; HBV*adr*4, Fujiyama *et al.*, 1983; HBV*ayr*, Okamota *et al.*, 1986; GSHV, Seeger *et al.*, 1984; WHV, Galibert *et al.*, 1982).

mobility in polyacrylamide gel, provides evidence for 3 proteins binding to this region including C/EBP (Faktor *et al.*, 1990; Ben-Levy *et al.*, 1989; Dikstein *et al.*, 1990a,b), and functional studies on a multimerised E site inserted 3 ´ to the β-globin gene have implicated transcription factors AP-1/Jun and C/EBP in activation of this heterologous promoter in association with the E site (Ben-Levy *et al.*, 1989; Dikstein *et al.*, 1990b; Faktor *et al.*, 1990).

Deletion of the E site from plasmid pHBV1004-B reduced HBsAg expression in HuH7 cells but not HepG2 cells. It is likely that the observed difference was due to the low level of C/EBP in HepG2 cells as determined by Western blot analysis and DNaseI footprint analysis (Friedman *et al.*, 1989). Dikstein *et al.* (1990a) noted that a 4 base change in the E site reduced transcriptional activity under the influence of multimerised mutant-E sites in Hep3B, Alexander and HeLa cells but not in HepG2 cells. This mutation was adjacent to but not part of the enhancer "core" consensus motif, but still blocked C/EBP binding (Dikstein *et al.*, 1990b). The effect of the block to C/EBP binding on transcription would not be observed in HepG2 cells which express only minimal C/EBP. (HeLa cells have been shown to express a similar DNA binding protein (Clark *et al.*, 1988)).

The data presented here are consistent with the hypothesis that HBsAg production is modulated upward by cellular factors that bind to the preS2/S promoter itself and to the HBV enhancer and that the C/EBP binding site within the enhancer is responsible for transcriptional activation of the preS2/S promoter rather than repression as observed by Pei and Shih (1990). The multimerised E binding site can serve as a target for transactivation in association with the β-globin promoter (Faktor and Shaul, 1990). However deletion of the E binding site from plasmid pHBV1004-B did not appear to alter the modulation of HBsAg expression by HBxAg supplied in *trans*. This suggests that other sites in the HBV genome may serve as HBxAg response elements. These may include sites outwith the HBV enhancer and the preS2/S promoter region as HBxAg supplied in *trans* was able to augment HBsAg secretion from cells transfected with plasmid pHBV1004-B in the presence of competing templates penHB and pspIIBX.

It is likely that the HBxAg reponse elements will be located at binding sites for cellular transcription factors as HBxAg itself does not show DNA binding potential (Faktor and Shaul, 1990; Jameel *et al.*, 1990; Twu *et al.*, 1990; Unger and Shaul, 1990; Wu *et al.*, 1990). Binding sites for cellular factors outwith the preS2/S promoter and enhancer include other C/EBP binding sites in the HBV genome including nucleotide positions 2312-2328 described above and 2924-2946 in the core promoter region (Lopez-Cabrera *et al.*, 1990). The latter site, however was present in the enhancerless core promoter/CAT constructs which were not (or only very weakly) transactivated by HBxAg (Spandau and Lee, 1988; Colgrove *et al.*, 1989; Siddiqui *et al.*, 1989). In addition the human serum albumin gene has a binding site consensus for C/EBP but is not transactivated by HBxAg (this study; Hu *et al.*, 1990), so it is unlikely that the effect of HBxAg is mediated through this cellular transcription factor. Other possible HBxAg response elements in the HBV genome include the glucocorticoid response element, which can mediate increased transcriptional activity of the SV40 early promoter in conjunction with the HBV enhancer in response to dexamethasone treatment (Tur-Kaspa *et al.*, 1988). The HBV genome also contains a highly conserved site that binds the transcription factor NF-κB. (J. Wells, personal communication; figure 4.13). This factor binding site serves as an HBxAg response element in the HIV1 LTR fused to the CAT gene (Siddiqui *et al.*, 1989; Twu *et al.*, 1989a,b). It will be interesting to delete these regions from the HBsAg expression plasmid pHBV1004-B to see if this affects the response to HBxAg supplied in *trans*.

The mechanism of transactivation by HBxAg and the possible role of this function in the development of HCC will be considered in detail in Chapter 6. An alternative

```
          1969                                                    2018
          |                                                       |
HBVadyw   CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCATTGTTTG GCTTTCAGTT
HBVayw    CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGTT
HBVadw    CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGCT
HBVadw2   CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGCT
HBVadr    CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGTT
HBVadr4   CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGTT
HBVayr    CATTTGTTCA GTGGTTCGTA GGGCTTTCCC CCACTGTTTG GCTTTCAGCT
GSHV      CCTTACTTCA ATGGTTAGGA GGAATTTCCC TCACTGTTTG GCTTTTGCTT
WHV       CCTTGCTTCA ATGGTTAGGA GGAATTTCCC TCATTGCGTG GTTTTTGCTT
Consensus C-TT--TTCA -TGGTT-G-A GG--TTTCCC -CA-TG--TG G-TTT----T

NFκB site from HIV1 LTR:      G GGACTTTCC(G/A)
NFκB site from Igκ enhancer:  G GGACTTTCCG
```

Figure 4.13: Comparison of NFκB-like sequence in the HBV clone used in this study (HBV*adyw*) with the sequence in other hepadnaviruses. Numbers represent nucleotide positions in HBV*adyw* (Appendix I). Consensus sequence and NFκB binding sites from the HIV1 LTR (Nabel and Baltimore, 1987) and the immunonglibin κ light chain enhancer (Sen and Baltimore, 1986) are shown. Methods of sequence comparison and references for hepadnaviral sequences are in the legend to figure 4.12.

mechanism to the possibility of direct protein-protein interactions with cellular transcription factors alluded to above, is the post-translational modification of cellular transcription factors by phosphorylation. There are several examples of alteration of transcription factor activity by phosphorylation (section 6A.4.1), and this possibility along with the heretofore unidentified source of the HBV virion associated kinase prompted my investigation into a potential protein kinase activity for HBxAg.

CHAPTER 5: Is HBxAg a protein kinase?

5.1 Introduction

A protein kinase (pk) activity has been described associated with 42 nm HBV particles, nucleocapsids extracted from virus particles (Albin and Robinson, 1980) and with core particles purified from whole cell extracts (Albin and Robinson, 1980; Feitleson *et al.*, 1982; Gerlich *et al.*, 1982) and cytoplasmic extracts (Petit and Pillot, 1985) of HBV infected hepatocytes. Incubation of these preparations with [γ-^{32}P]ATP resulted in phosphorylation of the core polypeptide. The reaction was enhanced in the presence of Mg^{++}, while inclusion of cAMP in the reaction mixture had no effect (Albin and Robinson, 1980). Quantitation of radioactive label incorporated into core particles revealed that only a small proportion of the 180 HBcAg subunits in the particle was phosphorylated (Gerlich *et al.*, 1982). In addition, treatment of labelled core particles with alkaline phosphatase did not remove the [^{32}P]phospho group (Gerlich *et al.*, 1982) indicating that the phosphate acceptor site and, by inference, the protein kinase itself are inside of the core particle. Acid hydrolysis of labelled core particles showed major phosphorylation sites on serine residues (Gerlich *et al.*, 1982; Feitleson *et al.*, 1982) contained within three tryptic peptides (Feitleson *et al.*, 1982). The labelled tryptic peptides for HBcAg and GSHV core antigen (GSHcAg), phosphorylated through an equivalent set of reactions, showed identical mobility upon two-dimensional fractionation. Consideration of the primary sequence of these two antigens identifies three identical tryptic peptides containing serine (HBcAg amino acid positions 155-157, 160-164, 168-172) derived from the protamine-like carboxyl-termini of HBcAg and GSHcAg, indicating phosphorylation of serine 155, serine 162 and (serine 168 and/or serine 172) in the HBcAg amino acid sequence.

The source of the endogenous pk activity found in HBV cores has not been identified. A pk activity is associated with numerous enveloped (Imblum and Wagner, 1974; Roux and Kolakofsky, 1974; Howard and Buchmeier, 1983; Stevely *et al.*, 1985; Roby and Gibson, 1986) and non-enveloped (Grubman *et al.*, 1981; Tsuzuki and Luftig, 1985, Ratka *et al.*, 1989) virus particles. In the case of herpes simplex virus type 1 and pseudorabies virus, the virion associated pk has the properties of casein kinase II and is most likely of cellular origin (Stevely *et al.*, 1985). However, a viral gene encoding a virion associated pk has been demonstrated in two cases. *In vitro* pk activity has been identified for the large polypeptide (L) of the virion associated transcriptive complex of two negative strand

RNA viruses, vesicular stomatitis virus (Chattopadhyay and Banerjee, 1987) and Sendai virus (Einberger *et al.*, 1990) with viral proteins as substrate. Thus a virally encoded pk activity for HBV would not be without precedent.

Endogenous polymerase activity was not required for endogenous pk activity of HBV core particles *in vitro*. Gerlich *et al.* (1982) showed that "heavy" core particles, which contain viral DNA and display polymerase activity, had less endogenous pk activity than "light" cores lacking viral DNA and associated polymerase activity when these variants were separated by isopycnic banding. Cytoplasmic core particles devoid of DNA polymerase activity were isolated from HBV infected liver and were positive for endogenous pk activity (Petit and Pillot, 1985). A model for encapsidation of viral pregenome mRNA suggests that the *pol*-gene product is incorporated into the capsid in a complex with the pregenome mRNA (Bartenschlager *et al.*, 1990). The detection of pk activity in core particles which have not encapsidated the viral polymerase indicates that the origin of the activity is not the viral polymerase polypeptide.

HBcAg particles purified from E. coli transformed with cloned DNA containing only the core ORF do not possess pk activity (Schlicht *et al.*, 1989a; this study) indicating that the core polypeptide itself is not the source of this enzymatic function. However, Serrano and Hirschman (1984) detected pk activity in purified HBeAg that had been secreted by buffalo rat liver cells stably transfected with the complete HBV genome. Despite employing several methods to dissociate other polypeptides from the preparation of HBeAg, it is possible that the material was contaminated with another polypeptide of viral or cellular origin accounting for pk activity.

Purified 22 nm HBsAg particles and filaments do not harbour endogenous pk activity (Gerlich *et al.*, 1982) indicating that the preS/S ORFs do not encode polypeptides with this enzymatic function. It remained to be investigated whether the X ORF encodes a pk activity.

5.2 Results

5.2.1 Plasmid constructions

Plasmid pHBcS111-156 (Stahl and Murray, 1989) contains the coding sequence for amino acid residues 111-156 of the major-S polypeptide (HBV nucleotide positions 1767-1905) fused through a 15 bp linker to the 3´ end of the coding sequence for a fusion

protein of β-galactosidase (amino acids 1-8)/HBcAg (amino acids 3-144). The coding sequences in this plasmid are derived from a modification of plasmid ptacHpaIIR2 (Stahl and Murray, 1989) engineered to contain a *Sma*I restriction site replacing the *Hind*III site contained in the parent plasmid. Transcription of protein coding sequences in these plasmids is under control of the *tac* promoter (de Boer *et al.*, 1983) which was constructed by replacing the -35 region of the *lac*UV5 promoter (Silverstone *et al.*, 1970) with the -35 region of the stronger *trp* promoter yielding increased transcription upon induction with IPTG. The HBsAg coding sequence in plasmid pHBcS111-156 was subcloned from a variant of plasmid pHBV130 (Gough and Murray, 1982) which contains a missense transition (HBV nucleotide 1794, C changed to T; HBsAg residue 120, Pro changed to Ser) relative to the sequence published by Pugh *et al.* (1986), creating a *Bam*HI restriction site.

Plasmid pHBcX contains the complete X ORF fused to the 3´ end of the coding sequence for a fusion protein of β-galactosidase (amino acids 1-8)/HBcAg (amino acids 3-144)/HBsAg (amino acids 111-120) and was constructed as follows (figure 5.1): plasmid pHBcS111-156 was linearised by digestion with *Bam*HI, filling in cohesive ends with DNA polymerase I (Klenow fragment) and digestion with *Sal*I. The X ORF was isolated from plasmid pHBV1004 by digestion with *Nco*I, filling in cohesive ends with DNA polymerase I (Klenow fragment) and digestion with *Sal*I. The resulting 622 bp fragment was ligated to the 4.6 kb, vector-containing fragment from pHBcS111-156. The integrity of the vector insert junctions and of the X-ORF was confirmed by DNA sequence determination (figure 5.2a).

Plasmid ptacX was constructed to express HBxAg fused to the carboxyl-terminus of β-galactosidase amino acids 1-8 under control of the *tac* promoter (figure 5.2b). Plasmid pHBcX was digested with *Eco*RI and *Nco*I and cohesive ends were rendered blunt by the action of DNA polymerase I (Klenow fragment). The resulting 4.7 kb fragment was isolated and recircularised by ligation.

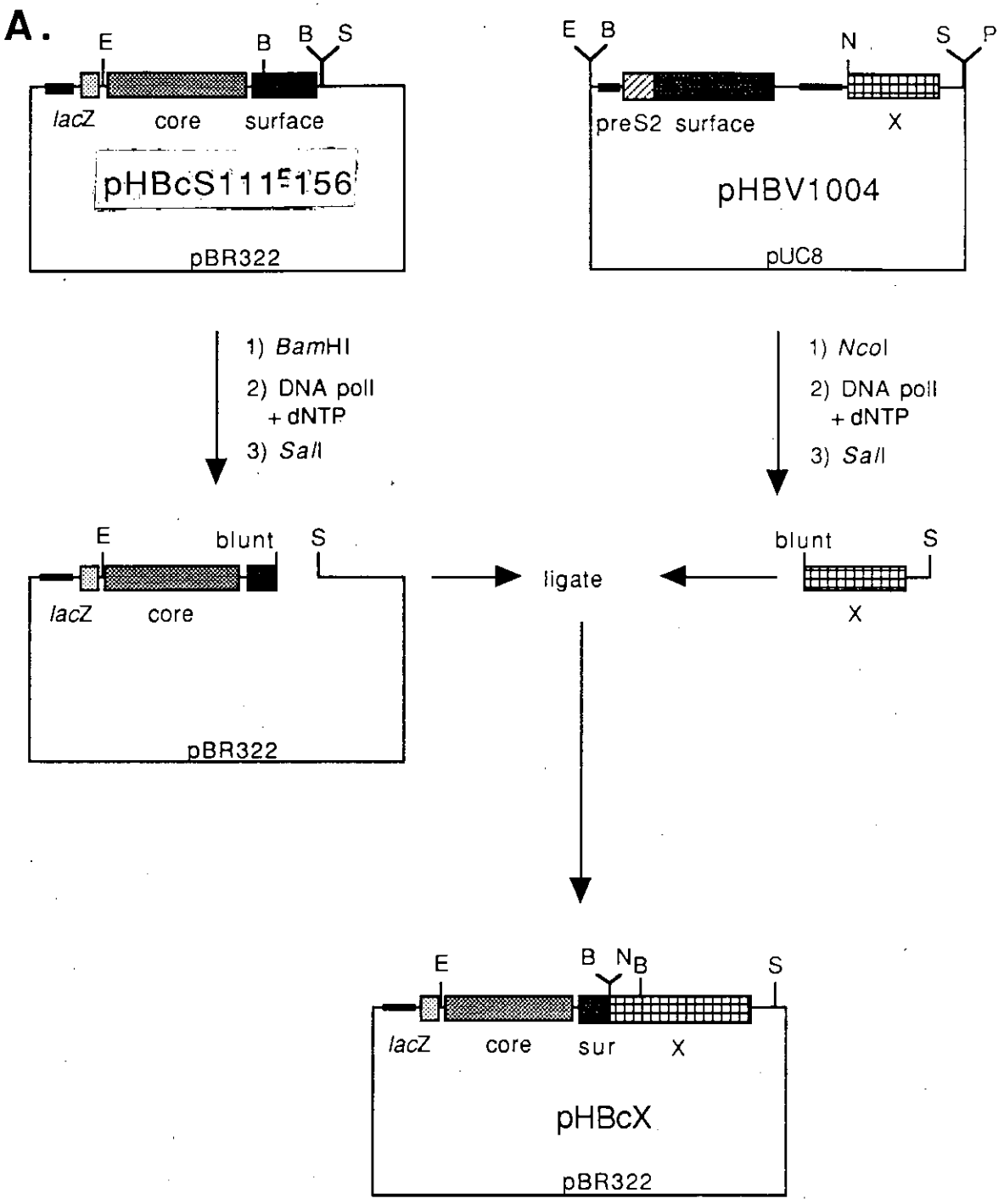
5.2.2 Purification of fusion protein encoded by pHBcX

Cells of *E. coli* strain RB791 harbouring plasmid ptacX were incubated with IPTG to induce transcription of the *lac*Z/X fusion gene (section 2B.10.10a). Induced cells were lysed by sonication, and insoluble material was removed by centrifugation. Both soluble and insoluble fractions were examined for HBxAg by immunoblotting which revealed a

FIGURE 5.1:

A) Construction of pHBcX. Details of construction are provided in the text (section 5.2.1). Thick lines represent transcriptional control regions; boxes represent the indicated ORFs; restriction endonuclease cleavage sites: B-*Bam*HI, E - *Eco*RI, N-*Nco*I, P-*Pst*I, S-*Sal*I.

B) Predicted amino acid sequence for HBcX. Single letter code is used for amino acids (aa). Numbers below boxes indicate amino acid position in the native protein. Also indicated are the HBV nucleotide positions of sequences contained in pHBcX. β-gal = β-galactosidase.


A.
E
B
B
S
lacZ
core
surface
pHBcS111E156
pBR322
E
B
N
S
P
preS2
surface
X
pHBV1004
pUC8
1) BamHI
2) DNA poll
+ dNTP
3) SalI
1) NcoI
2) DNA poll
+ dNTP
3) SalI
E
blunt
S
lacZ
core
pBR322
ligate
blunt
S
X
E
B
NB
S
lacZ
core
sur
X
pHBcX
pBR322

B.

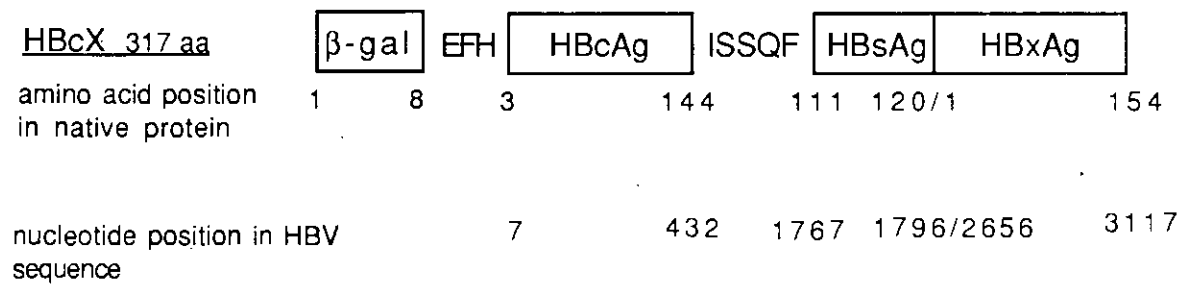
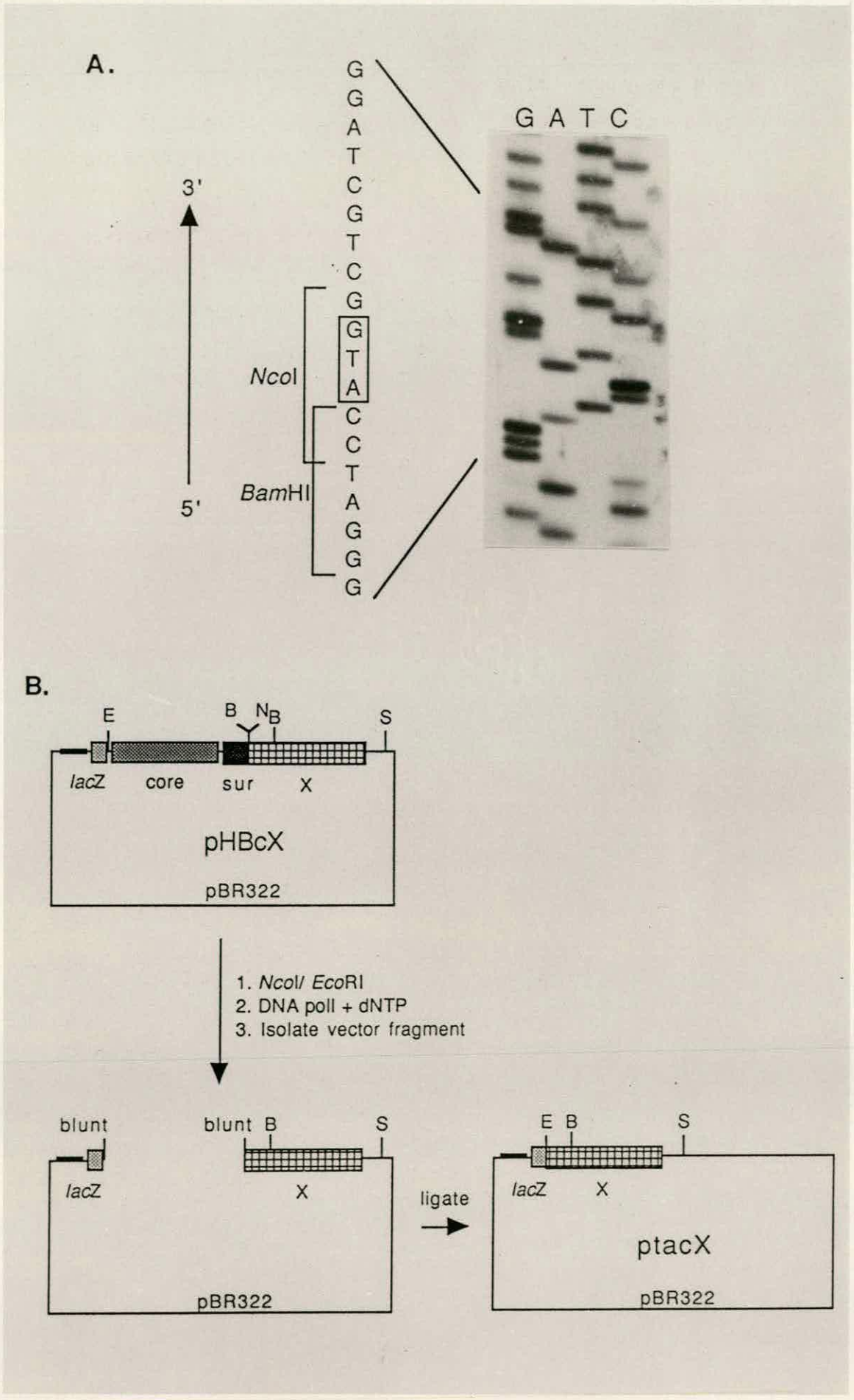

HBcX 317 aa
β-gal
EFH
HBcAg
ISSQF
HBsAg
HBxAg
amino acid position in native protein
1
8
3
144
111
120/1
154
nucleotide position in HBV sequence
7
432
1767
1796/2656
3117

FIGURE 5.2:

A) Nucleotide sequence of pHBcX at the junction of the 5′ end of the X ORF and the codon for amino acid residue 120 of the surface ORF. Plasmid pHBcX was digested with *Xba*I which cuts after HBV nucleotide 345 within the core ORF and with *Sal*I, and the resulting 745 bp fragment was ligated to M13mp19 linearised by digestion with *Xba*I and *Sal*I. Single stranded, recombinant, bacteriophage DNA was sequenced by dideoxynucleotide chain termination (Sanger *et al.*, 1977; section 2B.7) using the m13mp series -20 universal sequencing primer. The initiation codon of the X ORF is boxed; restriction endonuclease cleavage sites, *Bam*HI and *Nco*I, are indicated.

B) Construction of ptacX. Details of construction are provided in the text (section 5.2.1). Thick lines represent transcriptional control regions; boxes represent the indicated ORFs; "sur" indicates a fragment of the surface ORF; restriction endonuclease cleavage sites: B-*Bam*HI, E - *Eco*RI, N-*Nco*I, S-*Sal*I.


A.
G A T C
3'
5'
NcoI
BamHI
G G A T C G T C G G G T A C C T A G G G
B.
E
B N B
S
lacZ
core
sur
X
pHBcX
pBR322
1. NcoI/ EcoRI
2. DNA poll + dNTP
3. Isolate vector fragment
blunt
blunt B
S
lacZ
X
pBR322
ligate
E B
S
lacZ
X
ptacX
pBR322

very low level of fusion protein in the insoluble fraction and none in the soluble fraction (data not shown). To increase the yield of HBxAg produced in *E. coli* it was produced fused to the carboxyl-terminus of a fusion protein containing HBcAg amino acid residues 3-144.

Expression of the HBcAg/HBxAg fusion protein (HBcX) was induced in *E. coli* strain RB791 harbouring plasmid pHBcX (section 2B.10.10a). Analysis by immunoblotting of soluble proteins extracted from induced cells revealed a low level of fusion protein (<1% of total soluble protein), but a significantly higher level than had been obtained for expression of HBxAg encoded by plasmid ptacX (figure 5.3; lane "Cr"). The electrophoretic mobility of the fusion protein was slightly faster than calculated from the amino acid sequence (35.2 kD). This may be due to secondary structure in association with SDS or to disproportionate binding of SDS relative to the commercially obtained protein size standards.

As a first step in purification and characterisation of HBcX, proteins in the soluble fraction of *E. coli* cell extract were fractionated by gel filtration through Sepharose CL-4B. Fractions were assayed for the presence of fusion protein by immunodetection of proteins spotted onto nitrocellulose membrane (figure 5.7a), and proteins in positive fractions were fractionated by SDS page and analysed by immunoblotting. Figure 5.3 shows that HBcX was eluted in the void volume of the column indicating that it forms either particles or large aggregates. Insufficient fusion protein was obtained from "small-scale" preparation to continue purification; therefore, HBcX production was induced from a ten litre culture of *E. coli* strain RB791 harbouring plasmid pHBcX (section 2B.10.10b). Proceeding on the knowledge that HBcX formed particles or large aggregates, the purification procedure described by Stahl and Murray (1989) was followed initially. HBcX was sedimented from the soluble fraction of sonicated cell extract by ultracentrifugation. Proteins in the resuspended pellet were fractionated by gel filtration through Sepharose CL-4B and examined by SDS-PAGE (figure 5.5a). Fractions containing fusion protein were pooled and the product collected by ultracentrifugation. This preparation was observed by electron microscopy which revealed highly aggregated particles (figure 5.6). Individual particles were similar in appearance to HBcAg particles produced in *E. coli*.

Gallina and her colleagues (1989) showed that particles formed by HBeAg produced in *E. coli* could be dissociated by treatment with SDS or urea into dimeric form while particles formed by HBcAg could not be dissociated by these agents. This feature was exploited by Stahl and Murray (1989) to remove high molecular weight impurities from

FIGURE 5.3 Detection of HBcX following gel filtration through Sepharose CL-4B.

A) Protein samples were fractionated by SDS-PAGE in a 12.5% acrylamide gel and stained with Coomassie Blue.

B) Following SDS-PAGE fractionated proteins were transferred to nitrocellulose membrane and HBcX was detected by incubation with rabbit 98 anti-HBxAg serum (section 2A.5) diluted 1:2500. This antiserum had been pre-adsorbed against a lysate of *E.coli* strain RB791 (section 2B.10.7). Antibodies which bound to protein on the membrane were detected by incubation with goat anti-rabbit IgG serum conjugated to alkaline phosphatase and subsequent incubation with a colour producing substrate as described (section 2B.10.8).

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

C - 20 μg purified HBcAg.

Cr - Soluble fraction of sonicated lysate from *E. coli* strain RB791 harbouring plasmid pHBcX and incubated with IPTG.

4-9 - Number of fraction eluted from gel filtration of "Cr" through Sepharose CL-4B (30 μl of 3.3 ml fraction).

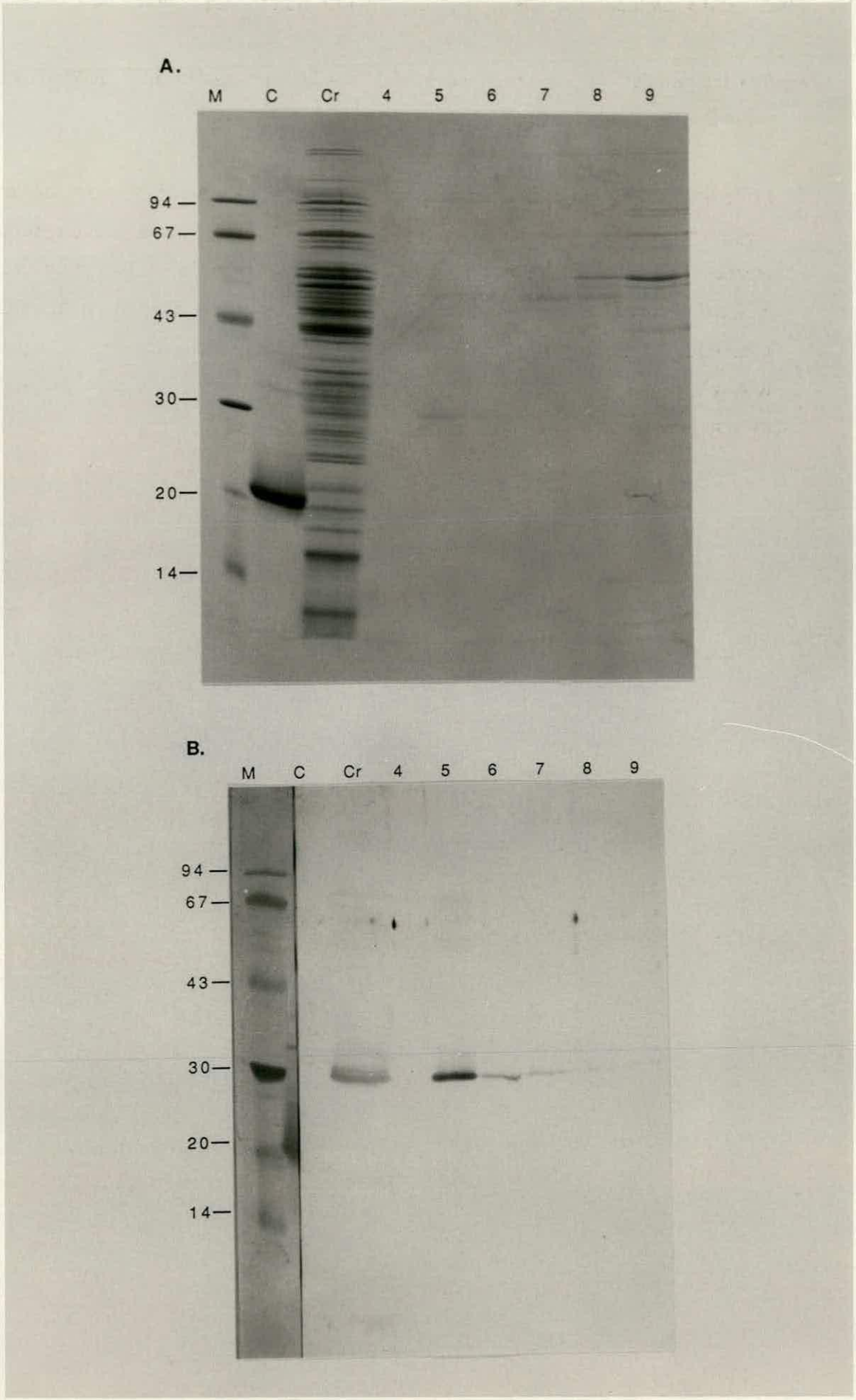

A.
M
C
Cr
4
5
6
7
8
9
94
67
43
30
20
14
B.
M
C
Cr
4
5
6
7
8
9
94
67
43
30
20
14

FIGURE 5.4 Electrophoresis of HBcX under non-reducing conditions.

A) Protein samples were fractionated by SDS-PAGE in a 12.5% acrylamide gel and stained with Coomassie Blue.

B) Immunoblot analysis carried out as described for figure 5.3b.

C) Immunoblot analysis as in "B" using rabbit 87 anti-HBcAg IgG preparation (section 2A.5) diluted 1:2500 for the primary incubation. This preparation had been pre-adsorbed against a lysate of *E. coli* strain RB791 (section 2B.10.7).

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

C - 20 μg HBcAg.

pBBx - Extract of *E. coli* strain DS410 harbouring plasmid pBBX (Pugh *et al.*, 1986) included as a positive control for rabbit 98 anti-HBxAg serum. The cross- reacting λcro/HBxAg fusion protein (17 kD) was present in panel B but was very faint and is not visible in the photograph.

CX1+DTT - HBcX following gel filtration through Sepharose CL-4B. 30 μl of fraction 4 (figure 5.5a, lane "4") was prepared by incubation in 1% (w/v) SDS, 60mM DTT for 5 minutes at 100°C.

CX1 - HBcX purified as above incubated in 1% (w/v) SDS for one hour at 60°C.

CX2+DTT - HBcX following gel filtration through Sephacryl S-200. 30 μl of fraction 4 (figure 5.5b lane "4") was prepared by incubation in 1% (w/v) SDS, 60mM DTT for 5 minutes at 100°C.

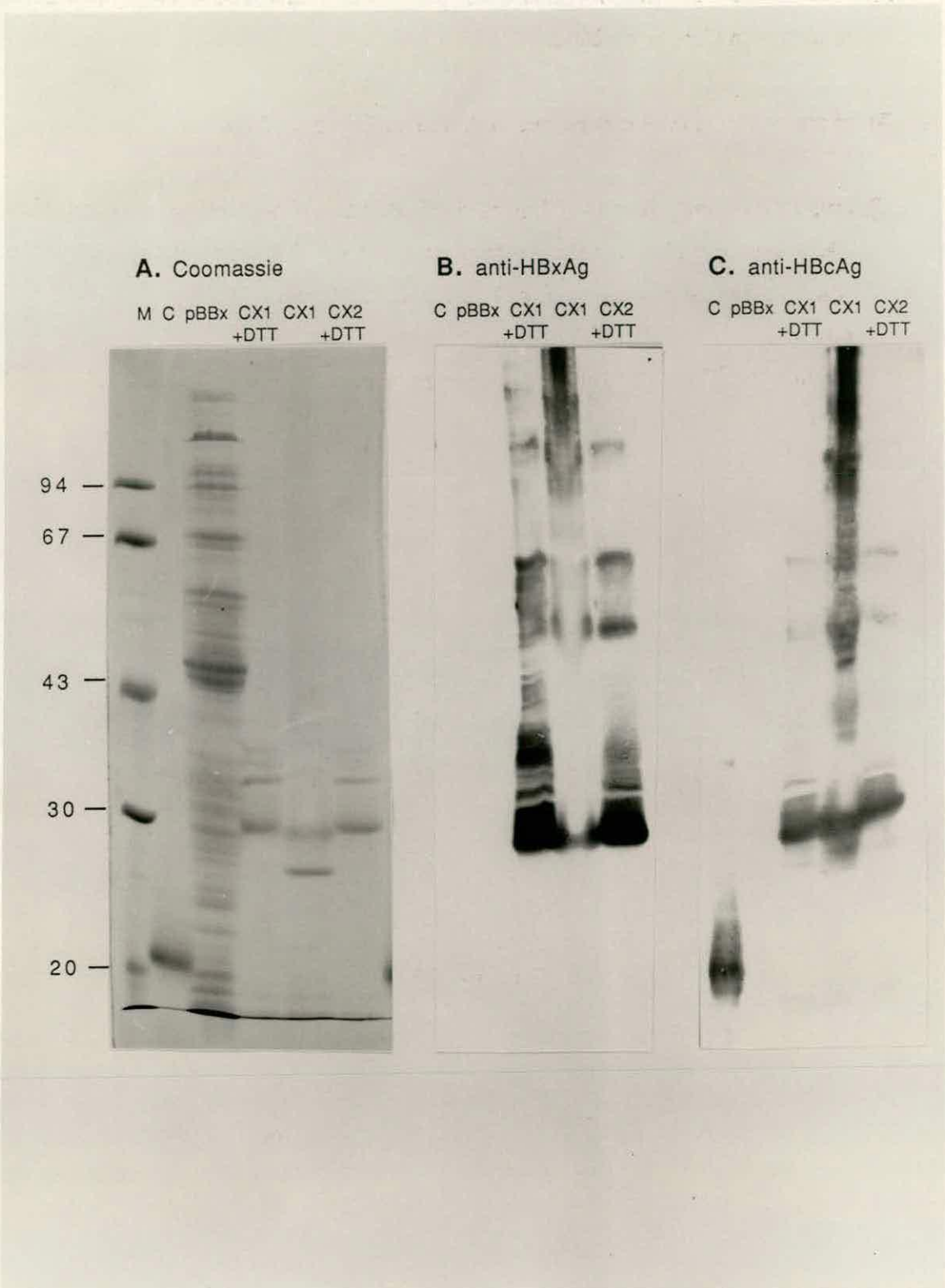

A. Coomassie
M C pBBx CX1 +DTT CX1 CX2 +DTT
94
67
43
30
20
B. anti-HBxAg
C pBBx CX1 +DTT CX1 CX2 +DTT
C. anti-HBcAg
C pBBx CX1 +DTT CX1 CX2 +DTT

FIGURE 5.5: "Large-scale" purification of HBcX.

Protein samples were fractionated by SDS-PAGE in 12.5% acrylamide gels and stained with Coomassie Blue.

A) Gel filtration through Sepharose 4B.

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

Cr - Soluble fraction of sonicated lysate from *E. coli* strain RB791 harbouring plasmid pHBcX and incubated with IPTG.

P - Resuspended pellet following ultracentrifugation of "Cr".

S - Supernatant following ultracentrifugation of "Cr".

1-9 - Number of fraction eluted from gel filtration of "P" through Sepharose CL-4B (30 μl of 3.3 ml fraction).

B) Gel filtration through Sephacryl-S200 following incubation of partially purified HBcX in 5M Urea

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

4Bp - Resuspended pellet following ultracentrifugation of HBcX purified by gel filtration through Sepharose CL-4B.

4Bs - Supernatant following ultracentrifugation of HBcX purified by gel filtration through Sepharose CL-4B.

1-8 - Number of fraction eluted from gel filtration of "4Bp" through Sephacryl S-200 (30 μl of 3.3 ml fraction).

C) Anion-exchange chromatography through DEAE-Sepharose.

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

C - 10 μg HBcAg.

S2p - Resuspended pellet following ultracentrifugation of HBcX purified by gel filtration through Sephacryl S-200.

S2s - Supernatant following ultracentrifugation of HBcX purified by gel filtration through Sephacryl S-200.

DEAE - 1/30 of total volume of resuspended pellet from ultracentrifugation of HBcX positive fractions eluted from a column of DEAE-Sepharose in a continuous gradient of 0 - 0.5 M NaCl.

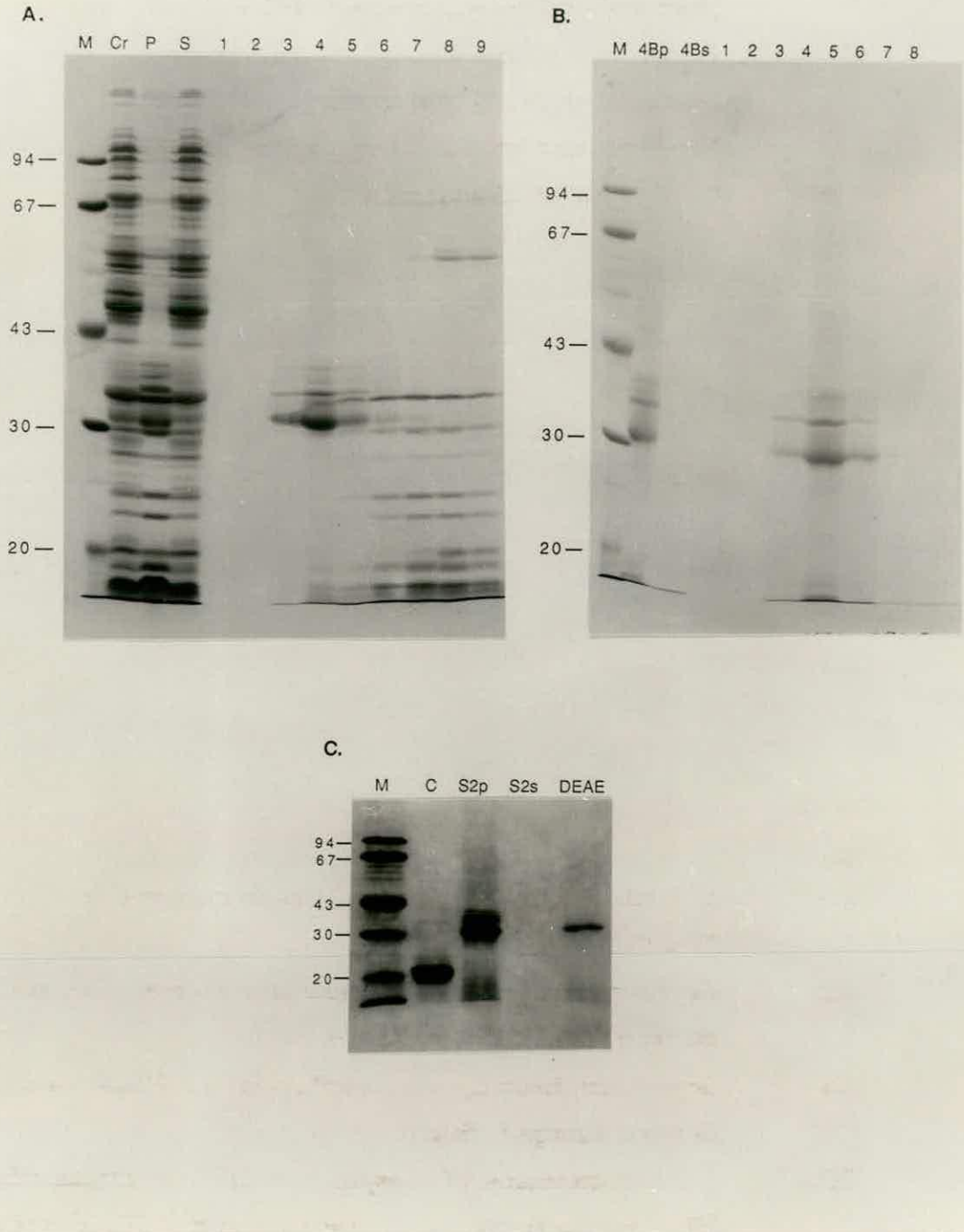
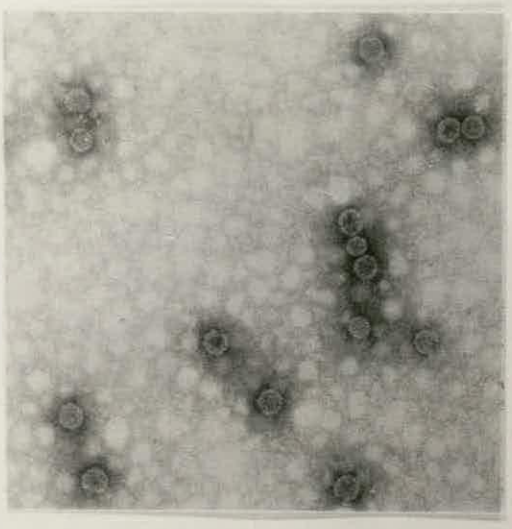

A.
M Cr P S 1 2 3 4 5 6 7 8 9
94
67
43
30
20
B.
M 4Bp 4Bs 1 2 3 4 5 6 7 8
94
67
43
30
20
C.
M C S2p S2s DEAE
94
67
43
30
20

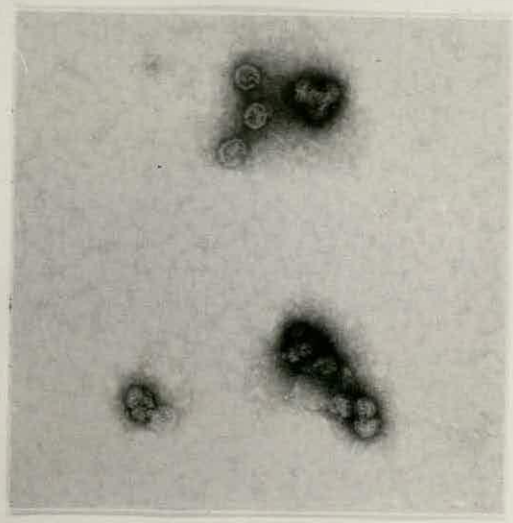
FIGURE 5.6: Observation of HBcX by electron microscopy.

Electron micrographs of HBcAg (A), HBcX following gel filtration through Sepharose CL-4B (B), and HBcX following anion-exchange chromatography through DEAE-Sepharose (C). Grids were prepared as described (section 2B.10.14). Magnification is 80,000 x.

A.



B.



C.

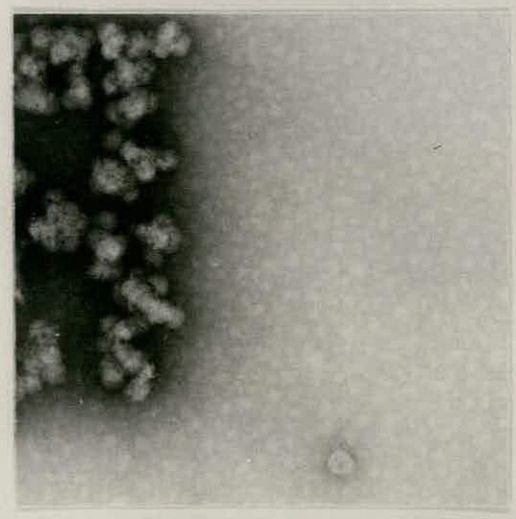


FIGURE 5.7: Immunoreactivity of HBcX

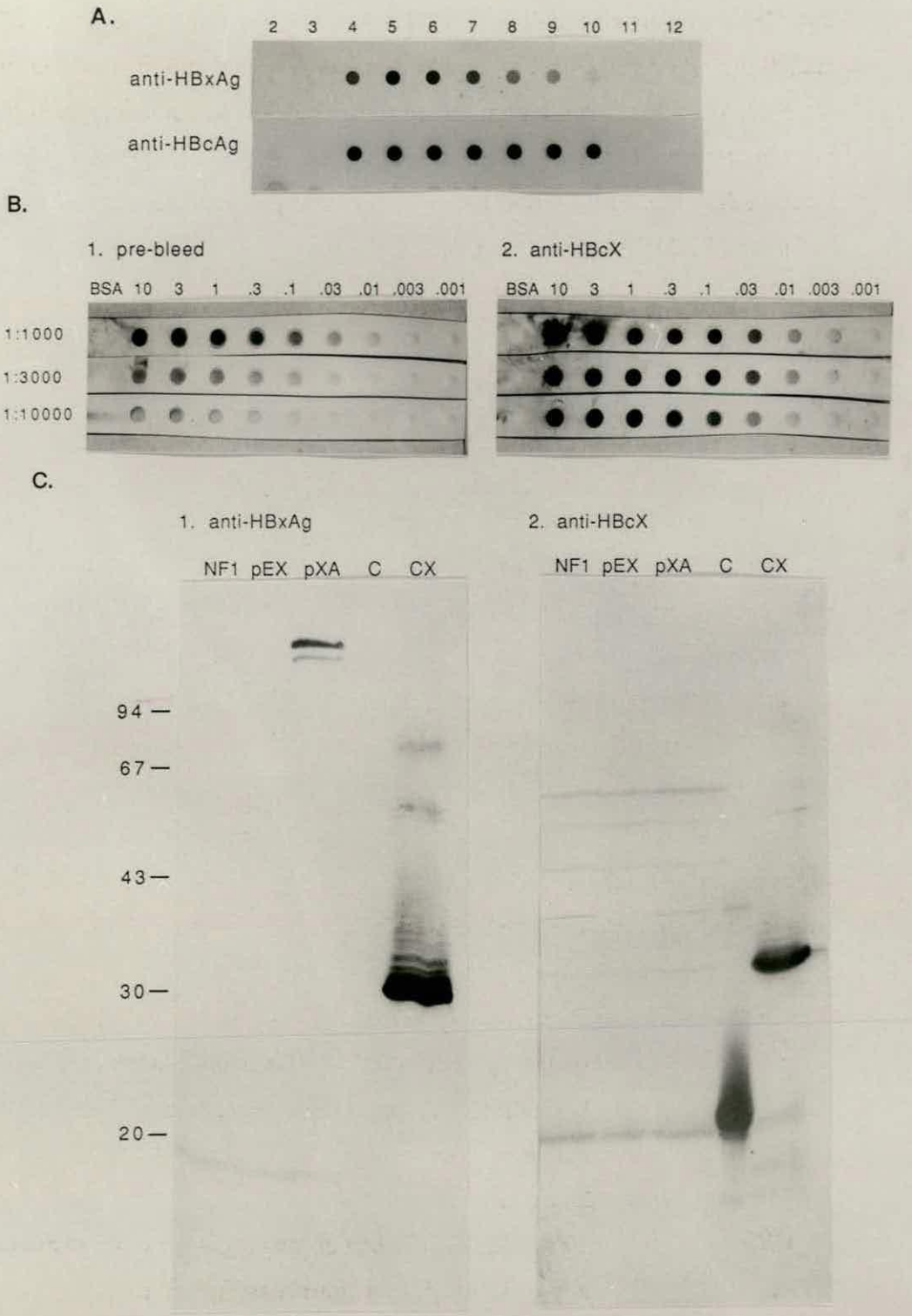
A) Detection of HBcX in fractions eluted from Sepharose CL-4B column. Number of fraction (3.3 ml) is indicated. 50 μl of each fraction was spotted onto nitrocellulose membrane as described (section 2B.10.6). Membrane was incubated with rabbit 98 anti-HBxAg serum or rabbit 87 anti-HBcAg IgG preparation (section 2A.5) diluted 1:2500 or 1:1000 respectively. Antibodies which bound proteins on the membrane were detected by incubation with goat anti-rabbit IgG serum conjugated to alkaline phosphatase and subsequent incubation with a colour producing substrate as described (section 2B.10.8). Both rabbit antisera had been pre-adsorbed against a lysate of *E. coli* strain RB791 (section 2B.10.7).

B) Detection of HBcAg by antisera from rabbit inoculated with HBcX. Numbers above each column indicate μg of HBcAg spotted onto nitrocellulose membrane as described (section 2B.10.6). "BSA" indicates 10 μg of bovine serum albumin spotted onto the membrane as a negative control. Strips of membrane were incubated in the indicated dilution of serum withdrawn prior to inoculation with HBcX (1) or following the third inoculation with HBcX (2) as described in section 5.2.3. Antibodies which bound protein on the membrane were detected as decribed in "A". Both sera had been pre-adsorbed as described in "A".

C) Proteins fractionated by SDS-PAGE in a 12.5% acrylamide gel were transferred to nitrocellulose membrane and incubated with rabbit 98 anti-HBxAg serum diluted 1:2500 (1) or anti-HBcX serum (described in "B") diluted 1:1000 (2). Antibodies which bound protein on the membrane were detected as described in "A". Both antisera had been pre-adsorbed as described in "A".

Lanes:

NF1, pEX - pXA	Extracts of *E. coli* strain NF1 alone (NF1), cells harbouring the vector plasmid (pEX) encoding β-galactosidase, and cells harbouring plasmid (pXA) encoding a β-galactosidase/HBxAg fusion protein.
C-	20 μg HBcAg
CX -	30 μg HBcX following chromatography through Sepharose CL-4B and concentration by ultracentrifugation.


A.
2 3 4 5 6 7 8 9 10 11 12
anti-HBxAg
anti-HBcAg
B.
1. pre-bleed
2. anti-HBcX
BSA 10 3 1 .3 .1 .03 .01 .003 .001
1:1000
1:3000
1:10000
C.
1. anti-HBxAg
2. anti-HBcX
NF1 pEX pXA C CX
94
67
43
30
20

fusion proteins containing HBcAg amino acid residues 3-144 by gel filtration of the dimeric form. When HBcX, purified by gel filtration through Sepharose CL-4B, was subject to SDS page under non-reducing conditions (denaturation in 1% SDS for 1 hour at 60°C) about 60% of fusion protein was dissociated into monomeric form, while larger forms and aggregated protein that did not enter the gel were also observed (figure 5.4). This suggested that HBcX particles might be dissociated by a chaotropic agent.

HBcX was incubated in 5M urea pH 9.6, and proteins were fractionated through Sephacryl S-200. Fractions eluted from the column were assayed for the presence of HBcX by SDS-PAGE, which showed that particles had eluted from the column in the void volume and had not been dissociated (figure 5.5b). The HBcX preparation was incubated in 8M urea pH 7.0 for one hour at 37°C, and proteins were fractionated through Sephacryl S-200 equilibrated with 8M urea. Once again, the HBcX particles were not dissociated and eluted from the column in the void volume (data not shown).

Two ion exchange resins were investigated for the ability to bind HBcX. The fusion protein was eluted in the void volume from a column of cation exchange resin, CM-Sepharose, and the eluted sample contained the same contaminants as had been present prior to chromatography (data not shown). However, HBcX bound to the anion exchange resin, DEAE-Sepharose, and a small fraction of the protein was eluted from the column in approximately 0.4M NaCl within a continuous gradient of 0 to 0.5M NaCl (figure 5.5c). Approximately two mg of partially purified HBcX was layered onto the column as estimated by Lowry protein assay, while only ~50 μg was recovered as estimated by staining with Coomassie Blue in parallel with a known amount of purified HBcAg after SDS-PAGE. The eluted protein appeared to have fewer contaminants than had been present prior to chromatography (figure 5.5c); contaminants representing ~35% of total protein in the starting material (estimated by densitometric analysis) were not observed in the eluted protein.

HBcX was purified from a second ten litre culture of *E. coli* RB791 harbouring plasmid pHBcX by gel filtration through Sepharose CL-4B followed by anion exchange chromatography through DEAE-Sepharose (section 2B.10.10c). DTT (10mM) was present throughout the purification procedure in an effort to minimise aggregation of HBcX particles. As before only a small fraction of input HBcX was eluted from DEAE-Sepharose in 0.4M NaCl. Additional protein was eluted with steps of 1M and 2M NaCl and with 8M urea.

The elution of additional HBcX in each step of buffer may represent varying

dissociation constants for protein with the adsorbent matrix due to several different sites of interaction on the protein surface. In addition, the adsorbent matrix may present a heterologous array of binding sites (Scopes, 1987). It is also possible that protein precipitated at the top of the column was resolubilised to greater degrees with each elution step, and that this precipitate was responsible for the low yields of protein eluted from the column.

5.2.3 Production of antiserum to HBcX

HBcX purified by chromatography through Sepharose CL-4B and DEAE-Sepharose was used to immunise a rabbit that had been pre-immunised 3 years previously with a particulate form of HBeAg produced in *E. coli*. HBcX (~5 μg) in Freund's complete adjuvant was injected intramuscularly followed by a second injection of HBcX (~5 μg) in Freund's incomplete adjuvant 30 days later. A third injection of HBcX (~5 μg) in Freund's incomplete adjuvant was administered 140 days after the initial injection. Blood from the rabbit was collected from an ear-vein prior to inoculation with HBcX and one week after each injection. Sera collected after the second and third injection with HBcX were assayed for the presence of antibodies to HBxAg by immunodetection of a β-galactosidase/HBxAg fusion protein (encoded by plasmid pXA; section 3.2.1) immobilised on nitrocellulose membrane. No antibodies directed against HBxAg were detected, however an increase in the titre of antibodies specific for HBcAg was detected by immunoblot analysis of serial dilutions of this antigen (produced in *E. coli*) spotted onto nitrocellulose membrane (figure 5.7b).

5.2.4 HBcX has no kinase activity

In vitro pk activity was assessed for HBcX at three different stages of purification: 1) following gel filtration through Sepharose CL-4B, 2) following elution from DEAE-Sepharose in 0.4M NaCl, and 3) following elution from DEAE-Sepharose in salt and urea steps. The reaction buffer included the components described by Albin and Robinson (1980) for optimal activity of the endogenous pk of HBV core particles. Purified HBcAg produced in *E. coli* was used as exogenous substrate as this polypeptide is the phosphate acceptor for the endogenous, HBV-associated pk. The incorporation of radioactive phosphate into protein was determined by absorption of labelled proteins to DE81 paper

and by autoradiography of labelled proteins fractionated by SDS-PAGE.

The ability of HBcAg to serve as phosphate acceptor in the reaction was shown by incubation with the catalytic subunit of cAMP-dependent protein kinase (PKA) which yielded up to 100% incorporation of [γ-^{32}P]ATP into HBcAg (figure 5.8a, "C+PKA"; figure 5.8b,c lane "CK"). HBcAg is not visible in the stain of total proteins (fig 5.8b, lane "CK") because the reaction mixture was diluted 300-fold before SDS-PAGE. Phosphorylation of HBcAg in the presence of [γ-^{32}P]ATP alone was not observed (figure 5.8a, "C"; figure 5.8b,c, lane "C") consistent with the result of Schlicht *et al.* (1989a) that HBcAg produced in *E. coli* does not have the capacity to autophosphorylate. In experiment 3 (figure 5.8a; column "C") the source of increased counts for HBcAg incubated with [γ-^{32}P]ATP alone is not known, because autoradiography of labelled proteins fractionated by SDS-PAGE showed that no radioactive phosphate has been covalently linked to HBcAg.

Incubation of HBcX (~1μg) with HBcAg in the presence of [γ-^{32}P]ATP did not result in phosphorylation of either protein (figure 5.8a, "CX+C"; figure 5.8b,c, lane "CX/C") indicating that the *X*-gene product produced in *E. coli* fused to part of HBcAg does not have pk activity for either autophosphorylation or for phosphorylation of HBcAg. In addition, HBcX did not serve as a substrate for PKA (figure 5.8a, "CX+PKA"; figure 5.8 b,c, lane "CX/K"), indicating that HBcAg is phosphorylated by this enzyme on serine residues within the carboxy-terminal 39 amino acids of HBcAg which are missing in HBcX. This region of HBcAg has two serine residues (positions 168 and 176) contained in consensus sites available for phosphorylation by PKA (Kemp and Pearson, 1990). The increased counts observed on one occasion (figure 5.8a, Experiment 3, "CX+PKA") for HBcX incubated with PKA in the presence of [γ-^{32}P]ATP was due to the phosphorylation of contaminating proteins as observed by autoradiography of reaction products separated by SDS-PAGE (figure 5.8c, lane CX/K).

5.2.5 Protein kinase activity in extracts of human hepatoma cells transiently expressing HBxAg

In vitro pk assays were carried out on crude freeze-thaw lysates prepared from hepatoma cells transiently transfected with a plasmid encoding HBxAg under control of its own promoter/enhancer (pHBV1004) or its corresponding X ORF frameshift mutant (pHBV1004-B), or from cells transiently transfected with a plasmid encoding HBxAg

FIGURE 5.8: Kinase assays with HBcX.

A) Average counts per minute (CPM) obtained from Cerenkov counting of kinase assay reaction mixtures absorbed in duplicate to DE81 paper and washed as described (section 2B.10.13) except in the case of "A total" which was unwashed. Contents of reaction mixtures were as follows:

A total - [γ-^{32}P]ATP alone in reaction buffer.

C+PKA - 20 μg HBcAg and O.5 μg catalytic subunit of c-AMP dependent protein kinase (PKA).

C - 20 μg HBcAg.

CX+C - ~1 μg HBcX and 20 μg HBcAg

CX+PKA - ~1 μg HBcX + 0.5 μg PKA

A - [γ-^{32}P]ATP alone in reaction buffer.

Results from four independent experiments are shown; N.D.= not done. HBcX preparations were from different stages of purification: 1) following gel filtration through Sepharose CL-4B; 2) following anion-exchange chromatography through DEAE-Sepharose and elution in continuous gradient of 0-0.5M NaCl; 3) as in "2" but from a second large scale preparation; 4) pooled protein eluted from DEAE-Sepharose by steps of increasing NaCl concentration and 8M Urea.

B) 30 μl of reaction mixture (except where indicated) was fractionated by SDS-PAGE in a 12.5% acrylamide gel and proteins were transferred to nitrocellulose membrane. Proteins on the membrane were stained with amido black. Arrow in "blank" lane indicates position of HBcX in lanes "CX/C" and "CX/K".

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

CK - HBcAg and PKA as in "C + PKA" above.

C - HBcAg as above; reaction mixture was diluted 1:300 prior to SDS-PAGE.

CX/C - HBcX and HBcAg as in "CX + C" above.

CX/K - HBcX and PKA as in "CX + PKA" above.

A - [γ-^{32}P]ATP as in "A" above.

C) Autoradiography of nitrocellulose membrane in "B". Lane CK, 7 hour exposure of film to membrane; lanes C, CX/C, CX/K and A, two day exposure.

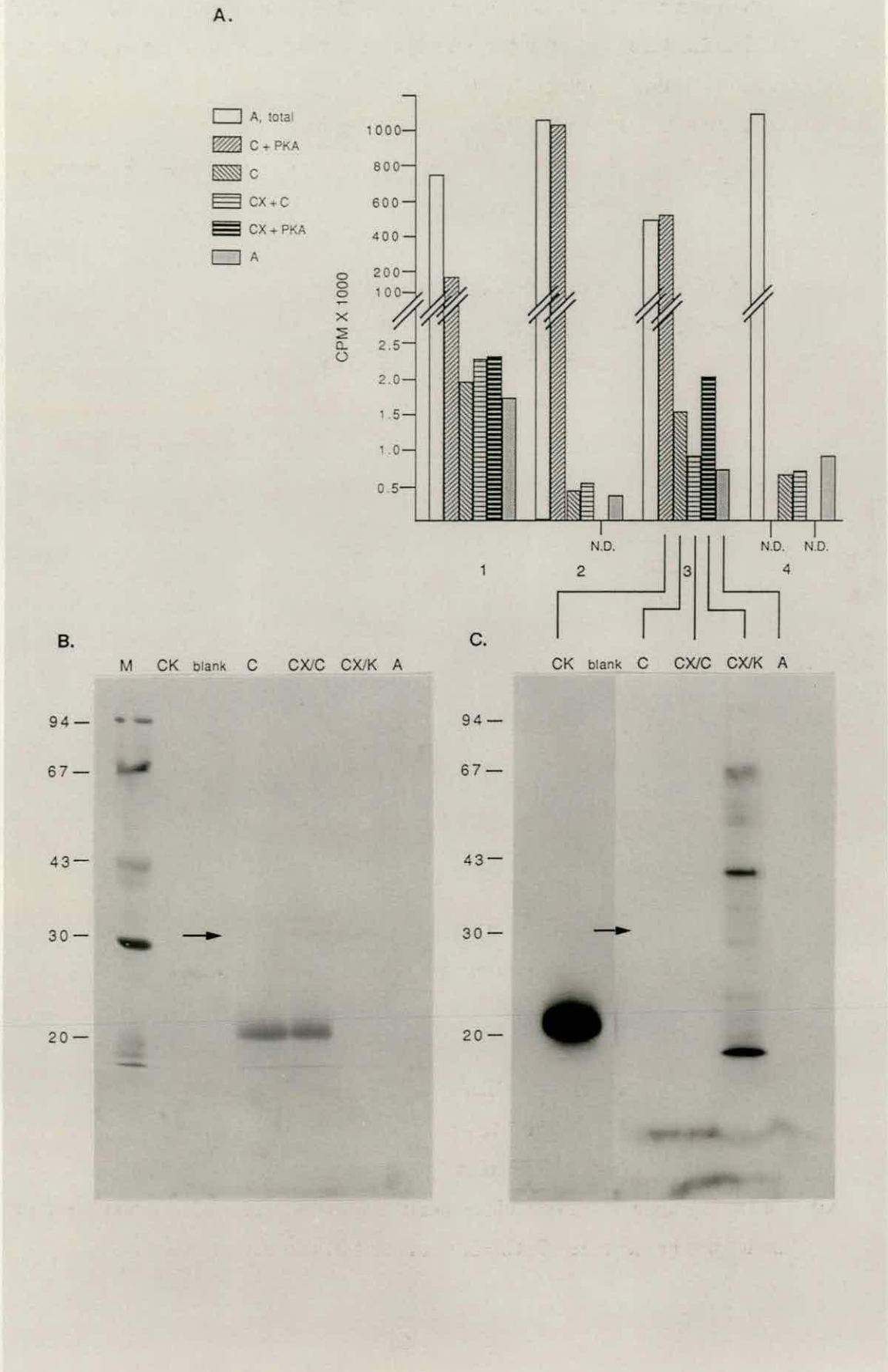

A.
A, total
C + PKA
C
CX + C
CX + PKA
A
CPM X 1000
1000
800
600
400
200
100
2.5
2.0
1.5
1.0
0.5
N.D.
N.D.
N.D.
1
2
3
4
B.
M
CK
blank
C
CX/C
CX/K
A
94
67
43
30
20
C.
CK
blank
C
CX/C
CX/K
A
94
67
43
30
20

TABLE 5.1 Kinase assays with mammalian cell extracts.

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Total	1058500	511250	1102900	467205
Background	397	733	930	362
4		10560		7301
B		11538		9447
X	18398	12654		8137
XB	19187	11884		8365
4αX		7739		4753
BαX		9250		5692
XαX		34843	37705	6842
XBαX		23383	19700	4336
XαX sup			13921	
XBαX sup			12765	
XD				643
XBD				608
XαC			38011	
XBαC			19095	

Values obtained from four independent experiments are shown. 10 μl of kinase reaction mixture (total volume, 50 μl) was absorbed to each of two pieces (1 cm^2) of DE81 filter paper. Except in the case of "total", which was unwashed, the filters were washed as described (section 2B.10.14) and Cerenkov counted. The value shown is the average value obtained for the duplicate filters. Reaction mixtures contained: "Total" and "Background", [γ-^{32}P]ATP alone; XαX sup and XBαX sup, soluble extract following immunoprecipitation as in XαX and XBαX; all others as in figure 5.9

under control of the SV40 early promoter/enhancer (pSV2HBX) or its corresponding frameshift mutant (pSV2HBX-B). Approximately 30 μg of total protein from the soluble fraction of each lysate was incubated with [γ-^{32}P]ATP and HBcAg added as an exogenous substrate. Similar pk activities were observed in soluble extracts from cells transfected with plasmids encoding HBxAg and those transfected with X-mutant plasmids (table 5.1 and figure 5.9; "4,B,X,XB").

Polyclonal anti-HBxAg serum was used to immunoprecipitate cross-reacting products from the soluble fraction of cell lysates. Protein kinase activity was determined for precipitated products immobilised on Sepharose beads coated with protein A. Similar

FIGURE 5.9: Kinase assays with mammalian cell extracts.

A) 30 μl of reaction mixture (section 2B.10.13) was fractionated by SDS-PAGE in a 12.5% acrylamide gel and transferred to nitrocellulose membrane. Proteins on the membrane were stained with amido black.

Lanes:

M - Molecular weight size standards (sizes indicated in kD).

4, B - Reaction mixture contained 30 μg of soluble protein from a freeze-thaw lysate of HuH7 cells transfected with 30 μg pHBV1004 (4) or 30 μg pHBV1004-B (B) per 90mm dish.

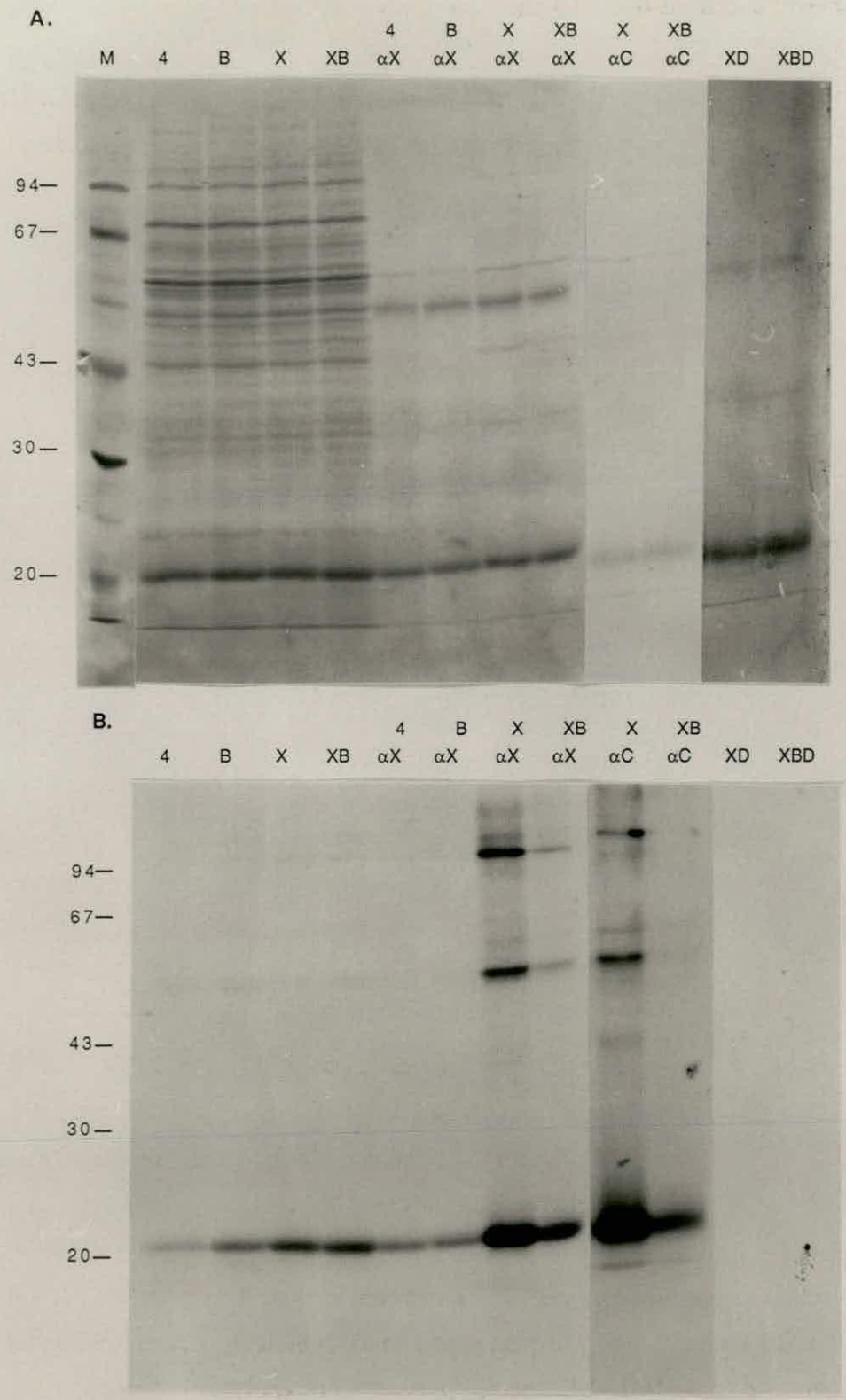
X, XB - Reaction mixture contained 30 μg of soluble protein from a freeze-thaw lysate of HepG2 cells transfected with 100 μg pSV2HBX (X) or 100 μg pSV2HBX-B (XB) per 90mm dish.

4αX, BαX, XαX, XBαX - Reaction mixture contained washed protein A beads following immunoprecipitation of cross-reacting products from soluble extract "4","B","X" or "XB" described above with rabbit 98 anti-HBxAg serum pre-adsorbed against a lysate of *E. coli* strain RB791 and diluted 1:500.

XαC, XBαC - Reaction mixture contained washed protein A beads following immunoprecipitation of cross-reacting products from soluble extract "X" or "XB" described above with rabbit 87 anti-HBcAg IgG preparation pre-adsorbed against a lysate of *E. coli* strain RB791 and diluted 1:500.

XD, XBD - Soluble fractions of freeze-thaw lysates "X" or "XB" described above were pre-incubated with 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid before immunoprecipitation with Rabbit 98 anti-HBxAg serum as described above.

B) Autoradiograph of nitrocellulose membrane in "A". Lanes 4, B, X, XB, 4αX, BαX, XαX, XBαX; 30 minute exposure of film to membrane. Lanes XαC and XBαC, two hour exposure; lanes XD and XBD, four hour exposure.


A.
M
4
B
X
XB
4 αX
B αX
X αX
XB αX
X αC
XB αC
XD
XBD
94
67
43
30
20
B.
4
B
X
XB
4 αX
B αX
X αX
XB αX
X αC
XB αC
XD
XBD
94
67
43
30
20

kinase activities were present in immunoprecipitates from cells transfected with pHBV1004 or pHBV1004-B, while greater kinase activity was detected in immunoprecipitates from cells transfected with plasmid pSV2HBX compared to pSV2HBX-B (table 5.1 and figure 5.9; 4αX, BαX, XαX, XBαX). However, immunoprecipitation of greater pk activity from extracts of cells expressing HBxAg under control of a heterologous promoter was not specific to this gene product, as the same result was observed when polyclonal anti-HBcAg serum was used for immunoprecipitation (table 5.1 and figure 5.9; XαC, XBαC). To reduce non-specific immunoprecipitation, soluble fractions were pre-incubated (10 minutes at 37°C) with detergents recommended for use in immunoprecipitation buffer (1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid; Harlow and Lane, 1988). As a control, soluble fractions without added detergent were pre-incubated for 10 minutes at 37°C. In the control samples, greater pk activity was immunopreciptated from cells transfected with pSV2HBX than with pSV2HBX-B as had been previously observed (table 5.1, Experiment 4, "XαX, XBαX"). However, incubation of soluble fractions with detergent, nearly abolished the non-specific immunoprecipitation of pk activity (table 5.1 and figure 5.9, "XD, XBD"). The low level of pk activity observed in these samples was equivalent for cells transfected with pSV2HBX or with the frameshift mutant.

5.3 Discussion

To examine if the X ORF of HBV encodes a protein kinase that could be responsible for the endogenous pk activity associated with HBV core particles, HBxAg was produced in *E. coli*. A very low yield of HBxAg was obtained when the antigen was fused at its amino terminus to the eight amino-terminal residues of β-galactosidase. Several recent studies have shown that heterologous viral sequences fused to the 3´ end of the coding sequence for HBcAg (up to amino acid residue 144) are produced in high yield in *E. coli* (Borisova *et al.*, 1989; Stahl and Murray, 1989). The fusion proteins spontaneously form particles making them easy to purify and immunogenic, eliciting antibodies to the heterologous polypeptide sequence. This system was exploited for the production of HBxAg in *E. coli* fused via a fifteen residue linker to the carboxyl-end of HBcAg residues 3-144.

In previous reports the largest polypeptide extension accommodated in HBcAg-like particles was 115 amino acids (Stahl and Murray, 1989). HBcX formed HBcAg-like

particles with an extension of 169 residues. However, unlike the fusion particles described by Stahl and Murray (1989), HBcX could not be dissociated by treatment with a chaotropic agent, although monomers were formed from a percentage of HBcX particles by incubation in 1% SDS at 60°C for one hour. It appears that some HBcX monomers are participating in particle superstructure by disulphide bonding.

Purified HBcX was not a substrate for PKA, while HBcAg was phosphorylated by this enzyme, indicating that the phosphate acceptor site in HBcAg in this reaction was located in the 39 carboxy-terminal residues. This is the same region phosphorylated by the endogenous pk activity of HBV core particles. The polypeptide sequences in HBcX do not contain a consensus sequence for phosphorylation by PKA and did not serve as a substrate for the enzyme. HBcX was not autophosphorylated in the presence of [γ-^{32}P]ATP alone and did not phosphorylate HBcAg added as an exogenous substrate.

It is pertinent to this discussion to consider whether the HBxAg segment contained in HBcX was located inside or outside of the chimeric capsid. If HBxAg was inside the particle, kinase activity would not have been accessible to exogenous substrates although nucleoside triphosphates should have been able to penetrate for assessment of autophosphorylation capacity. There is evidence that heterologous polypeptide sequences fused to HBcAg at amino acid residue 144 are presented on the surface of the particle. Chimeric capsids bound monoclonal antibodies directed against epitopes in the heterologous sequence (Borisova *et al.*, 1989), and, when used as immunogens, chimeric capsids elicited antibodies to the heterologous epitopes (Borisova *et al.*, 1989; Stahl and Murray, 1989). HBcX spotted onto nitrocellulose membrane cross-reacted with anti-HBxAg serum indicating exposure of HBxAg epitopes on the particle surface. However, a rabbit immunised with HBcX did not produce antibodies that cross reacted with denatured HBxAg epitopes presented on nitrocellulose membrane after SDS-PAGE, while antibodies to HBcAg were elicited. This may have been the result of a deficiency in the immune repertoire of the individual animal or a low response due to the age of the animal rather than a lack of HBxAg epitopes presented on the particle surface. Aggregation of HBcX particles may, in part, be due to interparticle disulphide bonds. If some of the 10 cysteine residues of HBxAg were exposed on the particle surface they may be available to form these bonds.

There are numerous examples of functional mammalian protein kinases produced in *E. coli*. These include serine/threonine kinases, for example, creatine kinase (Koretsky and Traxler, 1989) and the catalytic subunit of PKA (Slice and Taylor, 1989); and tyrosine

kinases, for example, v-*abl* (Pritchard *et al.*, 1989) and epidermal growth factor receptor (Farrow *et al.*, 1989). While it is thus possible for mammalian protein kinases produced in *E. coli* to fold in proper conformation for enzymatic function, the structural constraints of particle formation on HBcX may result in improper folding of the HBxAg segment. Therefore, the pk activity of HBxAg produced in human hepatoma cells was investigated.

Hepatoma cells were transfected with a plasmid encoding HBxAg under control of its native promoter/enhancer (pHBV1004) or under control of the SV40 early promoter enhancer (pSV2HBX) or with the corresponding X ORF frameshift mutant (pHBV1004-B or pSV2HBX-B). Higher pk activity was immunoprecipitated non-specifically from the soluble fraction of cells transfected with pSV2HBX than with pSV2HBX-B. The immunoprecipitation of greater pk activity from cells expressing HBxAg was abolished by incubation of soluble extracts with mild detergents. HBxAg was not detected by immunoblot analysis of extracts from cells transfected with pSV2HBX or pHBV1004. Indirect evidence for the presence of HBxAg in these cells is provided by detection of HBxAg specific mRNA in cells transfected with pSV2HBX and by the physiological consequence of increased HBsAg production in cells transfected with pHBV1004 compared to the corresponding frameshift mutant (Chapter 4). No HBxAg-specific pk activity was detected in these studies. The source of greater pk activity immunoprecipitated non-specifically from cells transfected with pSV2HBX compared to the frameshift mutant is unknown. It is unlikely to be any of the Ca^{++}-dependent or cyclic nucleotide-dependent protein kinases as these components were not included in the reaction buffer. In addition to Ca^{++}, protein kinase C (PKC) requires phosphatidylserine and an analog of diacylglycerol to function *in vitro* (Rodriguez-Pena and Rozengurt, 1984) and, therefor, would not have been active in this system.

In this study, pk activity was not detected for HBxAg produced in mammalian cells or produced in *E. coli* fused to HBcAg. A recent report from Wu and her colleagues (1990) describes a pk activity for HBxAg produced in *E. coli* or by transcription and translation *in vitro*. Both products had the capacity to transactivate transcription *in vitro* from the HIV1 LTR fused to the CAT gene. Incubation of purified HBxAg from both sources with [γ-^{32}P]ATP resulted in incorporation of radioactive phosphate into HBxAg itself or into histone HI added to the reaction mixture as an exogenous substrate. Zn^{++} was the only component of the reaction mixture used by Wu *et al.* that was not present in the mixture used in this study. Zn^{++} may be important for pk activity as has been demonstrated for the phosphorylation of poliovirus VP2 and VP4 by the virus-associated

pk (Ratka *et al.*, 1989).

Wu *et al.* describe several experiments that provide evidence against contamination of their preparations with a protein kinase other than HBxAg. A preparation of purified HBxAg derived from *E. coli* was fractionated by SDS-PAGE and the 16.5kd protein was eluted from the gel and renatured. The authors note that the pk activity observed with HBxAg purified in this manner was not observed using the same purification method for lysates of bacteria harbouring the vector plasmid without HBxAg coding sequences. They do not indicate, however, whether this negative result was obtained in the presence or absence of exogenous substrate. If one was not added, it is possible that the 16.5 kD X polypeptide served as substrate for a cellular kinase fractionated at the same place in the gel. Phosphorylation of the 16.5 kD purified protein was obtained following SDS-PAGE and transfer to nitrocellulose supporting the observation of an autophosphorylating kinase activity for HBxAg. The physiological significance of *in vitro* autophosphorylation of HBxAg is unkown. HBxAg produced in mammalian cells in culture does not appear to be phosporylated. When human embryonic cells, transiently transfected with a plasmid encoding HBxAg, were incubated in radioactive orthophosphate, only unlabelled p17 was immunoprecipitated from cell lysate with anti-HBxAg serum (Levrero *et al.*, 1990b).

In the study by Wu *et al.* HBxAg bound the ATP analog, p-fluorosulphonylbenzoyl 5´-adenosine (FSBA); ATP binding would be necessary for pk activity, and both *in vitro* phosphorylation of HBxAg and *in vitro* transactivation of the HIV1 LTR were blocked by preincubation with FSBA, implicating pk activity in transactivating function. The authors indicate that these functions were also abolished by denaturation of HBxAg in 6M guanidine chloride followed by removal of the denaturing agent. This is a curious result in light of the fact that HBxAg was initially purified from the insoluble fraction of *E. coli* extract by solubilisation in 7M urea and subsequent renaturation.

Wu *et al.* describe immunoprecipitation of radioactively labelled HBxAg from a preparation of 42 nm HBV particles, which were denatured following incubation in [γ-^{32}P]ATP. Unlabelled viral HBxAg could also bind FSBA. Acid hydrolysis of phosphorylated HBxAg from virus particles, or produced in *E. coli* or *in vitro* revealed incorporation of phosphate on serine and threonine residues. Complete tryptic digests of HBxAg from all three sources yielded identical labelled peptide maps. Eight tryptic peptides were labelled, i.e. every tryptic peptide of HBxAg (translated from the nucleotide sequence of the HBV subtype used in their study, *adw*2) containing serine or threonine (assuming complete digestion). Kinetic studies indicated that five molecules of phosphate

were transferred to each molecule of HBxAg during *in vitro* phosphorylation. Therefore, the phosphorylation of HBxAg observed by Wu *et al.* was non-specific with different residues phosphorylated in each HBxAg molecule.

Eukaryotic protein kinases constitute a large and diverse family of enzymes differing in cellular and subcellular distribution, regulatory control and substrate specificity, yet they show extensive sequence similarities throughout a catalytic core domain (Hanks *et al.*, 1988). In considering the amino acid sequence of HBxAg, Wu *et al.* conclude that it must be a novel protein serine/threonine kinase, as it is half the size of the catalytic domains of most protein kinases (300 amino acids or more) and does not contain consensus motifs conserved in eukaryotic protein serine/threonine kinases (Hanks *et al.*, 1988). Highly conserved features include the ATP-binding site comprising Gly-X-Gly-X-X-Gly followed by an invariant Lys 15-30 residues downstream, which appears to be directly involved in the phosphotransfer reaction. Protein serine/threonine kinases contain another conserved Lys ~100 residues further downstream which is diagnostic for this activity (tyrosine kinases have Arg or Ala at the equivalent position). Continuing ~10 residues downstream, the triplet Asp-Phe-Gly has been implicated in ATP binding, while ~20 residues further along, the triplet Ala-Pro-Glu lies near the catalytic site. A function has not been proposed for the conserved motif ~10 residues further downstream, Asp-X-Trp-X-X-Gly. HBxAg does not contain sequences that resemble any of these motifs and it is impossible to speculate at this point as to a mechanism for the proposed catalytic function. The cellular codon usage of HBxAg relative to the other HBV antigens (Miller and Robinson, 1986) suggests that it may have been acquired from the host genome. The primary structures of all eukaryotic protein serine/threonine kinases identified thus far show significant homologies to the conserved motifs in their catalytic domains and may have diverged from a single progenitor (Hunter, 1987). A eukaryotic kinase lacking these motifs must have diverged significantly or else be a completely novel sequence with convergent function. The latter suggestion is not inconceivable as prokaryotic protein kinases have evolved with a different primary structure, for example, *E. coli* isocitrate dehydrogenase kinase/phosphatase (Cortay *et al.*, 1988).

As well as not addressing the kinase activity of HBxAg produced in mammalian cells, Wu *et al.* do not address the ability of the HBxAg pk to phosphorylate HBcAg, which is known to serve as a substrate for the core particle associated pk. Whether HBcAg is phosphorylated by the capsid associated kinase *in vivo* is not known. The core antigen of both HBV (Roossinck and Siddiqui, 1987) and DHBV (Schlicht *et al.*, 1989a) produced

in human hepatoma cells in culture was phosphorylated in the protamine-like region of the carboxyl-terminus. During natural infection, phosphorylation of DHBcAg was associated with immature core particles isolated from the cytoplasm of DHBV infected hepatocytes, while nucleocapsids purified form DHBV virion were not phosphorylated (Pugh *et al.*, 1989). Phosphate groups on DHBV cytoplasmic core particles were removed by treatment with alkaline phosphatase. The susceptibility to alkaline phosphatase of HBcAg phosphorylated *in vivo* was not determined (Roossinck and Siddiqui, 1987). Phosphorylation of both DHBcAg (Pugh *et al.*, 1989) and HBcAg by the core particle associated pk is resistant to alkaline phosphatase indicating that *in vivo* and *in vitro* phosphorylating activities may be distinct. The presence of endogenous pk activity in DHBcAg particles argues against this activity being encoded by the *X*-gene product, as the DHBV genome lacks a homologous ORF.

Phosphorylation of proteins is a covalent means of regulating their activity, and the capsid proteins of numerous viruses are modified in this way *in vivo*. Phosphorylation of the poliovirus capsid proteins VP0, VP2, and VP4 may serve to destabilise the capsid for uncoating of the virus (Ratka *et al.*, 1989), while cleavage of $p65^{gag}$ polyprotein of Rauscher murine leukaemia virus and subsequent maturation of the virion may be related to phosphorylation (Naso *et al.*, 1979). Increased viral transcription was associated with phosphorylation of the influenza NP protein (Kamata and Watanabe, 1977), while phosphorylation of $p12^{gag}$ of Rauscher murine leukaemia virus was inversely proportional to RNA binding (Sen *et al.*, 1977). What physiological role might phosphorylation of HBcAg play?

Birnbaum and Nassal (1990) speculate that phosphorylation of HBcAg by the endogenous pk activity might release the viral genome from the capsid upon infection. Eckhardt *et al.* (1991) suggest that removal of the phospho group attached *in vivo* may play a role in nuclear localisation of HBcAg. In the duck model system mature core particles are not phosphorylated. In the absence of envelope synthesis, mature capsids localise to the nucleus resulting in amplification of covalently closed circular DHBV DNA (Summers *et al.*, 1990). During HBV infection immature (phophorylated?) nucleocapsids containing pregenome RNA and minus-strand DNA are localised to the cytoplasm (Gowans *et al.*, 1985; Miller *et al.*, 1984). Phosphorylation of HBcAg could regulate its transcriptional regulatory properties. While the effect of HBcAg on transcription of the HBV genome has not been investigated, this polypeptide can regulate transcription from the β-interferon promoter (Twu *et al.*, 1988; Twu and Schloemer, 1989).

In this study no pk activity was detected for HBxAg while a pk activity with unusual features has been described by Wu *et al.* (1990). The viral associated kinase responsible for endogenous activity has not yet been identified. A consideration of the implication of the proposed pk activity on a mechanism for transactivation by HBxAg will be considered in the next chapter.

CHAPTER 6: Mechanism of Transactivation by HBxAg and Potential Role for HBxAg in the Aetiology of HCC

6A Mechanism of Transactivation by HBxAg

6A.1 Some features of transcriptional control in eukaryotes

Initiation of transcription in eukaryotes is regulated by promoter sequences which contain clusters of short, defined DNA sequence motifs for binding specific protein factors. These transcription factors comprise two broad categories; general factors that are part of the RNA polymerase complex (the basal transcription apparatus) and are required for setting the transcription start site and for achieving a basal level of transcription, and regulatory factors that influence the efficiency of formation of active transcription complexes. The latter function can be modulated by a distant cluster of regulatory factor binding sites, an enhancer, which can operate in an orientation independent manner and was originally defined as a *cis*-acting regulatory sequence. Recently, evidence has been provided to show that a *cis*-linkage is not required for enhancer function; only a physical association between enhancer and promoter is necessary for interaction (Dunaway and Droge, 1989; Muller *et al.*, 1989). This observation supports the model that regulatory factors contact the basal transcription apparatus by looping out of the intervening DNA (reviewed by Atchison, 1988). Binding sites for a particular regulatory factor can be part of a promoter or an enhancer, and the consequences of factor binding may be positive or negative.

Regulatory factors can be composed of a polypeptide (or polypeptide homo-dimer) bearing both a DNA binding domain and a distinct activation domain required for transactivating or transrepressing function presumably by mediating protein-protein interactions. Examples of this type of factor include the yeast activator GAL4 or the mammalian transcription factors C/EBP and CREB (cAMP response element binding protein). Alternatively, activation and DNA binding domains may be provided by a complex of heterologous polypeptides (Ptashne and Gann, 1990; see below for examples).

Using the SV40 enhancer as a model system, it has been shown that there are distinct levels of organisation among regulatory factor binding motifs (Ondek *et al.*, 1988). In this system, enhancer "elements" are composed of two factor binding motifs (enhansons). The activity of enhansons within an element is sensitive to changes in spacing suggesting interactions between regulatory factors binding to adjacent sites, while elements within an enhancer are insensitive to spacing changes and may function in either orientation (Zenke *et al.*, 1986) suggesting participation in more distant protein-protein interactions.

Differential patterns of gene expression characteristic of distinct developmental stages or of different tissues may be attributed to variations in the level or activity of particular regulatory factors. This level, in turn, may be regulated by the transduction pathways for extracellular signals (see below). The complexity of transcriptional regulation in eukaryotic systems is manifest by the fact that transcriptional control regions may contain more than one binding sequence for a particular regulatory factor and/or the binding sequences for more than one type of factor. This modularity could allow regulation of transcription in response to diverse intracellular conditions (Dynan 1989). Additional complexity arises from identification of particular sequence motifs that can be bound by more than one type of transcription factor, for example, the sequence CCAAT and the octamer motif, and individual factors have been identified that bind divergent sequences, for example, C/EBP (reviewed by Johnson and McKnight, 1989). Therefore, multiple factors can influence transcription of a gene containing a particular binding motif in its regulatory region(s), and a single factor may evoke responses from genes with regulatory regions bearing different binding motifs. The level of transcription of a particular gene will be determined by an integrated association of regulatory factors within promoter and enhancer regions and the interaction of these factors with the basal transcription apparatus.

6A.2 Cellular factors mediate transactivation by HBxAg

The identification of many cellular transcription factors has occurred through investigation of viral transcriptional control regions. With the exception of pox viruses, all vertebrate DNA viruses and retroviruses rely on the cellular transcription machinery, and they have evolved regulatory sequences capable of recruiting cellular transcription factors. In addition, the genomes of most, if not all, DNA viruses encode regulatory proteins involved in the control of transcription of the viral genes. Two categories of viral transactivator proteins can be discerned based on whether they exhibit sequence-specific DNA binding (reviewed by Flint and Shenk, 1989). Those activators that bind directly to specific DNA sequences include the herpes simplex virus (HSV) ICP4 protein, the SV40 and polyoma virus large-T antigens and the Epstein-Barr virus BZLF1 protein. In contrast, the human T lymphotropic virus type 1 (HTLV1) $p40^{tax}$ protein, HSV VP16, adenovirus E1a and pseudorabies virus immediate early protein do not bind DNA directly.

The evidence available for HBxAg indicates that it falls into the second category of

viral transactivator. *In vitro* binding assays indicate that HBxAg produced in *E. coli* or by *in vitro* transcription and translation does not bind directly to DNA sequences that are targets for transactivation (Jameel *et al.*, 1990; Twu *et al.*, 1990, Wu *et al.*, 1990). There are several examples of viral or cellular transactivators that do not bind DNA themselves but as a complex with another polypeptide: VP16 exerts its effect at a specific octamer binding motif in a complex with the cellular Oct-1 protein (Triezenberg *et al.*, 1988); the proto-oncogene product c-Myc, interacts with a specific DNA sequence in a complex with the Max protein (Blackwood and Eisenman, 1991); and the 65 kD subunit of transcription factor NF-κB does not bind DNA itself but is part of the active sequence-specific factor in a complex with the 50 kD, DNA-binding subunit. Wu *et al.* (1990) cite unpublished data indicating that purified HBxAg derived from *E. coli*, when added to a nuclear extract from HepG2 cells, did not cause formation of any protein-DNA complexes with altered mobility in acrylamide gel compared to those formed using unsupplemented extract. This method has been used to demonstrate the ability of VP16 to form a sequence-specific DNA complex in association with Oct-1 (Preston *et al.*, 1988). The study by Wu *et al.* (1990) was limited to the HIV1 LTR and indicated that HBxAg does not form a strong complex with this sequence in the presence of cellular factors. However, it does not rule out a more subtle interaction between HBxAg and factors bound to DNA.

In vitro DNA binding studies indicate that HBxAg does not provide an integral part of a particular sequence specific transcription factor. This observation is supported by the diversity of targets susceptible to transactivation by HBxAg suggesting a less specific interaction. The recruitment of cellular factors by viral transcriptional control regions predicts that, if the function of a viral transactivator is mediated by cellular factors, it will have the capacity for transactivation of heterologous viral sequences as well as modulation of transcription of certain cellular genes. HBxAg displays this promiscuous capacity. Evidence for the involvement of regulatory (as opposed to general) cellular factors in the transactivation of genes transcribed by RNA polymerase II is provided by the observation that not all cell lines support transactivation of a particular target by HBxAg and not all known targets are transactivated in a particular cell line (Seto *et al.*, 1989; see table 4.1).

6A.3 Identification of factor binding sites that mediate transactivation by HBxAg

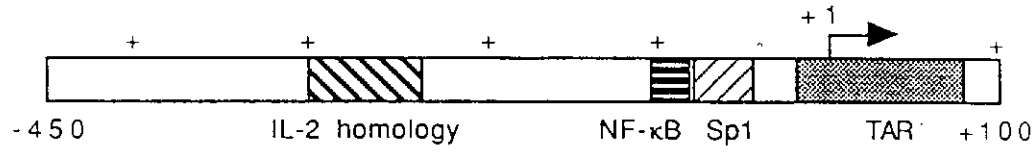
Investigation of the DNA sequences susceptible to transactivation by HBxAg suggests that its effect is mediated by more than one cellular factor, as no single recognition

sequence is found in all of the targets. It has been reported that HBxAg can transactivate the RNA polymerase III promoters of the adenovirus VA1 RNA or the $tRNA^{ala}$ gene contained in recombinant plasmid DNA (Aufiero and Schneider, 1990). Extracts of cells expressing HBxAg were shown to contain higher levels of active TFIIIC, a general factor in the RNA polymerase III transcription complex. This result would predict transactivation by HBxAg of cellular genes transcribed by RNA polymerase III which has not been thoroughly investigated.

Studies on the mechanism of transactivation of RNA polymerase II promoters by HBxAg have focused on deletion or mutation of individual regulatory factor binding sites from target promoters as well as on the generation of synthetic target promoters from cloned or synthesized factor binding sites. The HIV1 LTR fused to the CAT gene is a target for transactivation by HBxAg upon cotransfection of Jurkat T-lymphoma cells (Seto *et al.*, 1988, 1989). Several transcriptional regulatory regions have been defined within the HIV1 LTR including: the transacting response element (TAR) (nucleotide position -17 to +80 relative to the transcriptional start site) which is the target for transactivation by the HIV1 tat protein (Fisher *et al.*, 1986); binding sites for transcription factor Sp1 (-77 to -45) shown to bind the factor and to be important for activation of transcription (Jones *et al.*, 1986); the enhancer region (-104 to -81) consisting of two sequences with strong homology to the binding site for NF-κB in the immunoglobulin κ enhancer (this site confers inducibility of transcription from the HIV1 LTR by phorbol ester (Dinter *et al.*, 1987; see below) and purified NF-κB binds to and stimulates transcription from the HIV1 LTR *in vitro* (Kawakami *et al.*, 1988)); and a region homologous to the interleukin-2 (IL-2) distal and proximal control elements (-303 to -246) (figure 6.1a). Seto *et al.* (1988) showed that deletion from the 5′ end of the LTR, removing the region homologous to the IL-2 promoter, reduced the level of transactivation by HBxAg, while a further deletion of the NF-κB binding sites, leaving Sp1 sites and TAR intact, further reduced transactivation by HBxAg but still did not completely abolish it. Deletion of the LTR from the 3′end (to +41), removing about half of TAR abolished transactivation by HBxAg. The isolated NF-κB binding sites fused to the tk promoter and fused to the CAT gene were susceptible to transactivation by HBxAg but to a much lower level than the intact LTR. The authors conclude that multiple cis-acting sites within the HIV1 LTR are needed for HBxAg to exert its full effect.

The ability of HBxAg to function through multiple sites as described by Seto *et al.* (1988) was reminiscent of the activity of adenovirus E1a protein in transactivation of

A. HIV1 LTR



B. SV40 enhancer: one 72 bp repeat

FIGURE 6.1

A) Transcriptional regulatory regions defined within the HIV1 LTR. Plus signs denote 100 bp increments upstream and downstream of the transcription start site (+1). Individual regions are described in the text.

B) Transcription factor binding sites in the SV40 enhancer (one 72 bp repeat). Adapted from Seto *et al.* (1990).

adenovirus E2 and E3 genes, which can be mediated by any of several promoter elements (reviewed by Flint and Shenk, 1989). However, the similarity in the mechanisms for transactivation by HBxAg and E1a is called into question by the observation that a replication defective adenovirus mutant, which does not express the viral E1a gene, could not replicate in hepatoma cells expressing HBxAg, indicating that HBxAg could not compensate for the loss of E1a activity (Aufiero and Schneider, 1990). Furthermore, Twu *et al.* (1989a,b) and Siddiqui *et al.* (1989) have obtained conflicting results to those obtained by Seto *et al.* (1988) in examining transactivation of the HIV1 LTR fused to the CAT gene in HepG2 cells. Deletion of the NF-κB binding sites, leaving the Sp1 sites and TAR intact, completely abolished transactivation by HBxAg as did point mutations in the NF-κB binding sites within the complete LTR. In this system, the isolated NF-κB binding sites fused to the tk promoter fused to the CAT gene served as a target for transactivation by HBxAg that was nearly as efficient as the intact LTR (Twu *et al.*, 1989a). In addition, synthetic, multimeric NF-κB binding sites in association with the β-globin promoter (Twu *et al.*, 1989a) or fused to the SV40 early promoter (Faktor and Shaul, 1990) served as targets for transactivation of transcription of the CAT gene by HBxAg. The discrepancy in results could be due to the presence of different cellular factors (or factors in varying concentrations) in different cell lines.

NF-κB was originally detected as a nuclear protein that could bind a specific 10 bp sequence in the immunoglobulin κ light chain enhancer, and was present in B-cells only at the appropriate developmental stage for light chain expression (reviewed by Lenardo and Baltimore, 1989; Gilmore, 1990). Further investigation revealed that NF-κB is present in the cytoplasm of all non-B-cells tested as an inactive complex with an inhibitor protein, IκB. Treatment of cells with various agents such as phorbol esters results in the release of active NF-κB from its inhibitor and subsequent translocation of the active factor to the nucleus. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are stable analogs of diacyglycerol (DAG). DAG is a catabolic product of inositol phosphate metabolism induced by ligand binding to cell-surface receptors, and it is responsible for activation of protein kinase C (PKC). Hence, NF-κB is not only developmentally regulated, as in B-cells, but is also involved in inducible gene expression through phosphorylation of IκB by PKC (Ghosh and Baltimore, 1990).

It is unclear whether HBxAg is affecting the PKC inducible pathway of NF-κB. In HepG2 cells, the HIV1 LTR was transactivated by HBxAg to the same extent in the presence or absence of PMA suggesting that they function through the same pathway

which is stimulated to its full extent by HBxAg (Twu *et al.*, 1989a). In contrast, treatment of Jurkat cells with PMA in the presence of HBxAg gave multiplicative induction of transcription from the HIV1 LTR relative to that observed with either activator alone indicating distinct pathways for induction of NF-κB by PMA and HBxAg (Seto *et al.*, 1990). While other pathways for activation of NF-κB have been observed, for example, the PKC-independent induction of NF-κB activity by tumour necrosis factor α (Meichle *et al.*, 1990), it seems unlikely that a single activator, HBxAg, would exert its effect on a single factor through different pathways even in different cell lines.

Many of the transcriptional regulatory regions that are targets for transactivation by HBxAg contain binding sites for NF-κB. These include the SV40 enhancer (Sen and Baltimore, 1986; Chiu *et al.*, 1987), the HIV1 LTR (Kawakami *et al.*, 1988), the β-interferon promoter (Lenardo *et al.*, 1989), and the MHC class I promoter (Baldwin and Sharp, 1988). Other targets, however do not contain NF-κB binding sites, notably the HBV enhancer (personal observation), the HTLV1 LTR (Dingwall, 1991), the promoter of MHC class II gene HLA-DR (Hu *et al.*, 1990) and the c-*myc* promoters (Wingender, 1988), indicating that HBxAg can exert its effect through other cellular factors. Mutational analysis was carried out on the SV40 enhancer (one of the 72 bp repeats, figure 6.1b) fused to the human metallothionein promoter fused to the CAT gene and transiently expressed in CV1 monkey kidney cells (Seto *et al.*, 1990). Mutations in the binding sites for transcription factors AP-1 or AP-2 reduced basal transcriptional activity as well as the extent of transactivation of transcription by HBxAg while not abolishing this effect. When both sites were mutated, transactivation was reduced further. Mutations in the binding site for octamer binding factors or NF-κB/AP-3 reduced basal transcription but did not affect the extent of transactivation by HBxAg. Multimers of synthetic AP-1 or AP-2 binding sites fused to the human metallothionein promoter fused to the CAT gene were susceptible to transactivation by HBxAg, while a multimeric, mutant AP-2 site showed a reduced response. Further evidence for the mediation of transactivation through the cellular factor AP-2 was provided by transfection of the plasmid construction bearing multimeric AP-2-binding sites into *Drosophila* Schneider cells which are devoid of AP-2 activity. Cotransfection with an HBxAg expression plasmid did not yield transactivation while cotransfection with HBxAg and AP-2 expression plasmids gave a significant response. AP-2 binding sites are located in the c-*myc* promoter (Wingender, 1988) and consensus sequences for AP-2 binding are located in the promoters of MHC class II genes including HLA-DR (Benoist *et al.*, 1990).

Support for the involvement of AP-2 in mediating transactivation by HBxAg may be inferred from the results of Spandau and Lee (1988) who observed that transactivation of the SV40 early promoter/enhancer complex in CV1 cells could be blocked by cotransfection with a plasmid expressing SV40 large T-antigen (Tag) or by carrying out transactivation assays in COS cells which express Tag from integrated SV40 DNA. Mitchel *et al.* (1987) have shown that, through direct protein-protein interaction, Tag inhibits the ability of AP-2 to bind DNA. Tag did not block transactivation of the RSV LTR (Spandau and Lee, 1988) which lacks AP-2 binding sites (Wingender, 1988), implicating AP-2 in mediating transactivation of the SV40 early promoter/enhancer and indicating, once again, that the function of HBxAg can be mediated by more than one cellular factor.

The HIV1 LTR contains a sequence motif which resembles an AP-2 binding site (-95 to -88), however, no transactivation was observed in HepG2 cells of deletion mutants in which this site remained intact. (Twu *et al.*, 1989b; Siddiqui *et al.*, 1989). Multimers of this site in association with the β-globin promoter did not serve as a target for transactivation by HBxAg in HepG2 cells (Twu *et al.* 1989a). In apparent contrast, SV40 Tag inhibited transactivation of the HIV1 LTR by HBxAg in monkey kidney cells (Seto *et al.*, 1989). Perhaps the block to transactivation of the HIV1 LTR by Tag could be accomplished by a mechanism other than inactivation of AP-2. It is possible that Tag also interacts with and inactivates NF-κB although this has not been reported.

Support for the involvement of AP-1 in mediating transactivation by HBxAg may be inferred from the results of Faktor and Shaul (1990) who showed that multimers of the HBV enhancer E binding sites can serve as a target. This site was defined by its ability to bind C/EBP, but as described above (section 4.3), the presence of C/EBP binding sites does not confer susceptibility to transactivation by HBxAg. The sequence of the E site also contains homology to the consensus sequence for an AP-1 binding site. As with NF-κB, the activity of AP1 is modulated by phorbol esters (Jones *et al.*, 1988). Transcription from a plasmid bearing multimeric E binding sites was activated by phorbol ester implicating AP1 in interaction with this site where it may also function to mediate transactivation by HBxAg.

The HBV E site contains sequence homology to the HTLV1 $p40^{tax}$ response element (taxRE) which consists of three repeats of 21 bp in the viral LTR). $p40^{tax}$ does not bind to taxRE but stimulates transcription from this site as well as from the heterologous HBV E site (Faktor and Shaul, 1990). Additional similarity between HBxAg and $p40^{tax}$ is

indicated by the ability of $p40^{tax}$ to stimulate transcription from promoters containing NF-κB binding sites including the HIV1 LTR (reviewed by Dingwall, 1991). Unlike HBxAg, however, $p40^{tax}$ is exclusively a nuclear protein, and it could not transactivate the HTLV1 LTR in which the enhancer fragment (-350 to -55) was in reverse orientation (Rosen *et al.*, 1985). This modified LTR sequence, as well as the intact HTLV1 LTR, served as targets for transactivation by HBxAg (Wollersheim *et al.*, 1988; Zahm *et al.*, 1988; Siddiqui *et al.*, 1989).

Goodarzi *et al.* (1990) examined a large segment of the HBV enhancer for its ability to mediate transactivation by HBxAg in association with the SV40 early promoter. Consistent with the results presented in Chapter 4, mutations in the E site did not alter the ability of this enhancer/promoter complex to serve as a target for transactivation by HBxAg, while mutations in the EP site (see figure 1.7) reduced stimulation of transcription by HBxAg. This region of the HBV enhancer can compete with the polyoma virus enhancer for binding of a factor termed EF-C. Therefor, it appears that this factor may also mediate transactivation by HBxAg.

6A.4 Mechanisms for alteration of the activity of cellular transcription factors

TFIIIC, NF-κB, AP-1, AP-2 and EF-C have all been reported as having potential to mediate transactivation by HBxAg, and it is probable that this list is not exhaustive. A variety of mechanisms can be envisioned to explain the increase in transcriptional activity mediated by a cellular factor in the presence of HBxAg. HBxAg could simply stimulate the gene(s) encoding particular factors, thereby increasing their concentration in the cell. If a factor was in limiting concentration before HBxAg was present, increasing its abundance would stimulate promoters containing the corresponding binding site. This proposed mechanism does not answer the question of how HBxAg transactivates but merely shifts the question to a different target promoter. It is unlikely that HBxAg functions in this manner as it would require *de novo* protein synthesis, and transactivation by HBxAg produced in *E. coli* or in human hepatoma cells has been observed *in vitro* (Aufiero and Schneider, 1990; Wu *et al.*, 1990).

Two of the ways in which a transactivator might increase the activity of a particular transcription factor are by enhancing its ability to bind DNA or by improving its ability to interact with other regulatory factors or with the basal transcription apparatus. Either of these modulations could be achieved by, a) post-translational modification of the

transcription factor, or b) direct interaction between the activator protein and the transcription factor (figure 6.2). Any proposed mechanism for the function of HBxAg must seek a common pathway whereby several distinct cellular factors are regulated coordinately. In case "a" HBxAg must activate a modification pathway that alters the function of several different factors, while in case "b", HBxAg itself must be able to interact with different cellular factors.

6A.4.1 Post-translational modification

Phosphorylation of transcription factors has been shown to regulate their DNA-binding activity or to modulate their ability to activate transcription without affecting their DNA-binding capacity. Phosphorylation of c-Jun (a component of transcription factor AP-1) inhibits DNA binding while the binding activities of factors E2F and E4F, which are induced by adenovirus E1a, are increased by phosphorylation (Boyle *et al.*, 1991; and references therein). Phosphorylation of IκB releases NF-κB from a cystolic complex of the two proteins and allows NF-κB to enter the nucleus and bind DNA (reviewed by Lenardo and Baltimore, 1989). The DNA-binding activity of CREB (Gonzalez and Montminy, 1989) and yeast heat shock factor (Sorger and Pelham, 1988) is not affected by phosphorylation, however, their activity is increased by this modification which may be attributed to enhanced interaction with other elements of the transcription apparatus. In the case of CREB, this effect is not simply due to the increase in negative charge by phosphorylation as replacement of the phosphorylated serine residue with an aspartate or a glutamate residue created an inactive polypeptide (Gonzalez and Montminy, 1989).

It has been claimed that HBxAg itself possesses a protein serine/threonine kinase activity resulting in autophosphorylation of serine residues (Wu *et al.*, 1990; section 5.3), although no such activity could be detected in this study. In the experiments of Wu *et al.*, HBxAg derived from *E. coli* could transactivate the HIV1 LTR *in vitro* when added to the template in the presence of HepG2 cell nuclear extract. This effect could be abolished by pre-treatment of purified HBxAg with an analog of ATP known to block pk activity. Transactivation *in vitro* could not be mediated by NF-κB as the factor would not be present in a nuclear extract of HepG2 cells. The capacity of HBxAg to transactivate *in vitro* appears to contradict previous results reported by the same investigators indicating that *de novo* protein synthesis was required for transactivation of the HIV1 LTR in a T-cell line treated with *E. coli* derived HBxAg by protoplast fusion (Twu *et al.*, 1990).

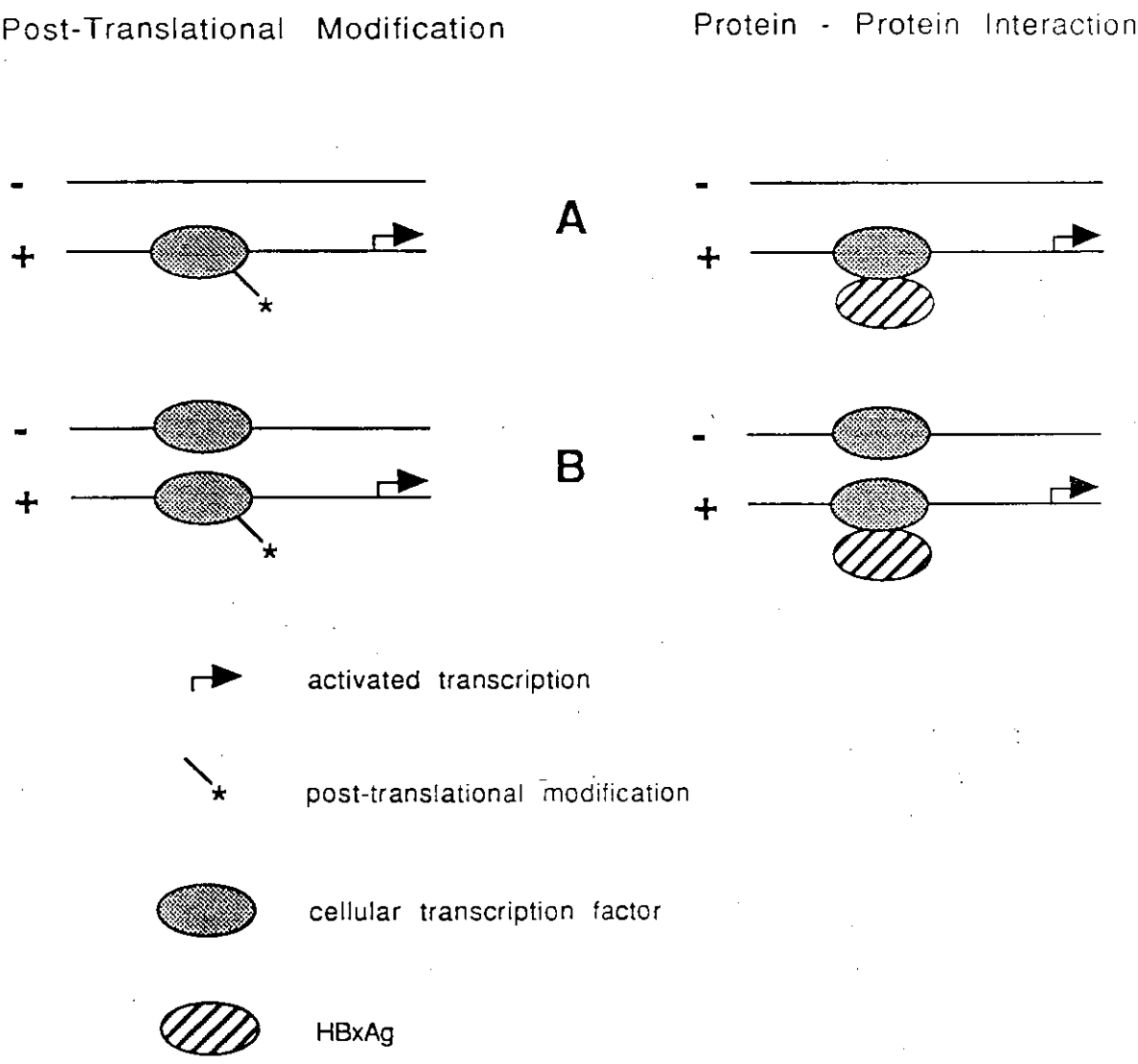


FIGURE 6.2 Mechanisms of transactivation

Schematic diagram depicting alternative models for the mechanism of transactivation by HBxAg. In each case, two transcriptional control regions are depicted in the presence (+) or absence (-) of HBxAg. The arrow denotes transcriptional activation in the presence of HBxAg.

A) Post-translational modification in the presence of HBxAg (left) or direct interaction with HBxAg (right) enhances DNA binding by a cellular regulatory factor.

B) Post-translational modification in the presence of HBxAg (left) or direct interaction with HBxAg (right) does not alter the DNA-binding capacity of a cellular regulatory factor but enhances its ability to interact with other components of the transcription complex.

Adapted from Nevins (1989).

However, if transactivation *in vivo* were being mediated by NF-κB, *de novo* protein synthesis may have been needed over the time course of the experiment to maintain the concentration of this factor. Hohmann *et al.* (1991) have shown that activated NF-κB (released from IκB) has a very short half-life and needs to be replaced by *de novo* protein synthesis in order to maintain the activated state. Active NF-κB in cells pre-treated with PMA was completely degraded within 2.5 hours of treatment with cycloheximide. The experiment by Twu *et al* (1990) involved treatment of cells with cycloheximide for 48 hours.

Wu *et al.* (1990) note that protein complexes with the HIV1 LTR DNA were formed more efficiently after incubation of the LTR DNA fragment in HepG2 cell nuclear extract supplemented with HBxAg derived from *E. coli* than after incubation in unsupplemented extract. This effect, too, was abolished by pretreatment of HBxAg with the ATP analog suggesting that the pk activity of HBxAg enhances the binding of cellular transcription factors (other than NF-κB) with the HIV1 LTR.

If the HBxAg kinase itself is responsible for mediating transactivation of transcription, its substrates must include all of the cellular factors shown to be involved in this function. To date, the only cellular substrate described for HBxAg pk activity is histone H1 (Wu *et al.*, 1990). Alternatively, the HBxAg kinase may activate a single cellular protein kinase for which the transcription factors are substrates. One candidate cellular kinase would be PKC. The activity of AP-1, AP-2 (reviewed by Jones *et al.*, 1988), and NF-κB (reviewed by Gilmore, 1990) is activated by phosphorylation following induction of signal transduction pathways activating PKC. TFIIIC is activated by phosphorylation in the presence of adenovirus E1a protein (Hoeffler *et al.*, 1988), although the source of kinase activity is yet to be determined. The modification of EF-C by phosphorylation has not been reported. In order to investigate the effect of PKC on HBV transcription and its involvement in transactivation by HBxAg, cells transfected with HBsAg expression plasmids (with or without an intact X-ORF) could be treated with phorbol esters to induce PKC or, alternatively, treated with specific inhibitors of PKC.

While the post-translational modification of cellular transcription factors remains a possibility, the balance of evidence currently favours a model postulating that HBxAg regulates transcription through protein-protein interactions.

6A.4.2 Protein-protein interactions

The concentration dependence observed for transactivation by HBxAg suggests that it functions by direct protein-protein interactions with cellular transcription factors (section 4.3). This hypothesis is supported by the construction of sequence-specific transcriptional regulatory factors by fusing HBxAg to the DNA-binding domain of a transcriptional control protein. Fusion of HBxAg to the DNA-binding domain of the bacterial repressor protein, LexA, conferred upon HBxAg the ability to activate transcription from a reporter plasmid bearing a lexA operator sequence fused to the human metallothionein promoter fused to the CAT gene (Seto *et al.*, 1990). Fusion of amino acid residues 1-143 of HBxAg to the DNA binding domain of transcription factor C/EBP from rat liver, significantly improved the ability of HBxAg to activate transcription from a construct bearing the HBV E site (C/EBP binding site) in association with the β-globin promoter and the tk promoter (Unger and Shaul, 1990). These results indicate that HBxAg has a transcriptional activation domain which is brought into the transcription complex by association with DNA-binding cellular factors. The activation domain of HBxAg was localised to amino acid residues 110-143, as a protein containing only amino acid residues 1-109 of HBxAg fused to the DNA-binding domain of C/EBP showed greatly reduced activation of transcription from the reporter plasmid bearing C/EBP binding sites (Unger and Shaul, 1990).

How might HBxAg function in the RNA polymerase II transcription complex? The interaction of the activation domains of cellular regulatory factors with the basal transcription apparatus may occur through different mechanisms (reviewed by Sawadogo and Sentenac, 1990). TFIID is a general transcription factor which binds to the "TATA box" present in most eukaryotic promoters and directs assembly of the basal transcription apparatus. Some factors (for example, CREB) bind DNA cooperatively with TFIID suggesting direct protein-protein interactions, while others (Sp1, NF1) do not. TFIID may not be relevant in the transactivation of transcription directed by the preS2/S promoter of HBV, as this promoter does not contain a "TATA box", and it remains to be shown whether promoters of this type utilise TFIID. However, the interaction of transcription factors with TFIID provides an interesting model for connections between regulatory factors and the basal transcription apparatus. It is becoming increasingly clear that there are adaptor proteins that can mediate this function (reviewed by Lewin, 1990; Ptashne and Gann, 1990), and HBxAg may serve as one of these adaptor molecules with an ability to

interact with several regulatory factors.

Fusion proteins directing a transcriptional activation domain to a particular DNA sequence (as described above for HBxAg) provide useful tools to investigate whether viral transactivators that do not bind DNA serve as a direct or indirect link with the basal transcription apparatus. VP16 is thought to provide the activation domain as part of a complex with the cellular DNA-binding protein Oct-1 and there is now evidence that an adaptor is required to link this complex to the basal transcription apparatus. Gal4-VP16 consisting of the DNA-binding domain of yeast Gal4 protein fused to the activation domain of VP16 activates transcription *in vitro* from a template containing a Gal4 binding site. Activation can be abolished by further purification of one of the yeast nuclear fractions comprising the extract for *in vitro* transcription without affecting the level of basal transcription. The potential of Gal4-VP16 to transactivate can be restored by addition of a distinct fraction of yeast nuclear extract which has no effect on basal transcription (Flanagan *et al.*, 1991). The requirement for an adaptor protein for interaction with the basal transcription apparatus has also been described for transcription factor Sp1 which binds a specific DNA sequence and contains an activation domain (Pugh and Tjian, 1990). It is becoming clear that the activation domains described for many regulatory transcription factors do not necessarily contact the basal transcription apparatus but may contact an adaptor protein.

Flanagan *et al.* (1991) site unpublished work indicating that an assay has been developed identifying an adaptor required for activity of NF-κB. It would be interesting to see if HBxAg could serve this function. Adaptor function would require two domains involved in protein-protein interaction, one contacting the regulatory factor and the other contacting the basal transcription apparatus. The latter of these regions may be the activation domain described by Unger and Shaul (1990). If this model is correct a truncated HBxAg polypeptide (amino acid residue 1-109) may interfere with the function of wild type HBxAg by sequestering cellular factors in the absence of the capacity to interact with the basal transcription apparatus. Experiments of this type have not yet been reported.

6B Potential Mechanisms for the involvement of HBxAg in the Development of HCC

The potential for HBxAg to contribute to the development of liver-cell neoplasia has been discussed above (section 1.6). In this section, the mechanism whereby HBxAg or a form of HBxAg altered by fusion to cellular sequences may cause neoplastic growth is considered. The biochemical functions that have been identified for known proto-oncogenes (the cellular homologs of viral transforming genes) fall into three categories (reviewed by Bishop, 1991): 1) GTPases involved in transduction of signals at the plasma membrane (for example, Ras); 2) protein kinases that are activated to phosphorylate substrates in response to extracellular or intracellular signals; 3) transcription factors regulating gene expression. Deregulation of any of these functions, for example, by introduction of a viral gene product into the cell, may lead to neoplasia. HBxAg has the potential to contribute to the development of HCC in its proposed capacity as protein serine/threonine kinase or as a transcriptional activator (figure 6.3a).

There are two known proto-oncogene products (Raf and Mos) with protein serine/threonine kinase activity whose expression is associated with cellular proliferation. The c-*mos* product may be responsible for phosphorylation of the cell cycle control protein, MPF (maturation promoting factor) (O'keefe *et al.*, 1989). Mitogenic stimulation of cells yields an increase in phosphorylation of the c-*raf* product and an associated increase in pk activity (Morrison *et al.*, 1988). The viral *raf* product can induce transcription of the c-*fos* oncogene most likely by phosphorylating a factor that regulates transcription of this gene (Jamal and Ziff, 1990). The components of the transcription factor AP-1 (c-*fos* and c-*jun* products) may serve as substrates for Raf pk activity (Wasylyk *et al.*, 1989).

Activation of PKC is associated with treatment of cells with tumour promoting agents (see section 6A.3), and, as discussed above (section 6A.4.1), transcription factors serve as substrates for PKC. It is becoming evident that activation of cellular transcription factors is an important step in cellular proliferation leading to neoplasia, and HBxAg may participate in the development of HCC by activation of transcription through either mechanism described above (section 6A.4).

The identification of proto-oncogene products that are transcriptional regulatory factors, for example, c-Fos, c-Jun, c-Rel (reviewed by Lewin, 1991) and c-Ets (Wasylyk *et al.*, 1990), indicates that aberrant expression of these factors themselves can cause neoplasia. Increased expression of a transcription factor, or alteration of the factor itself

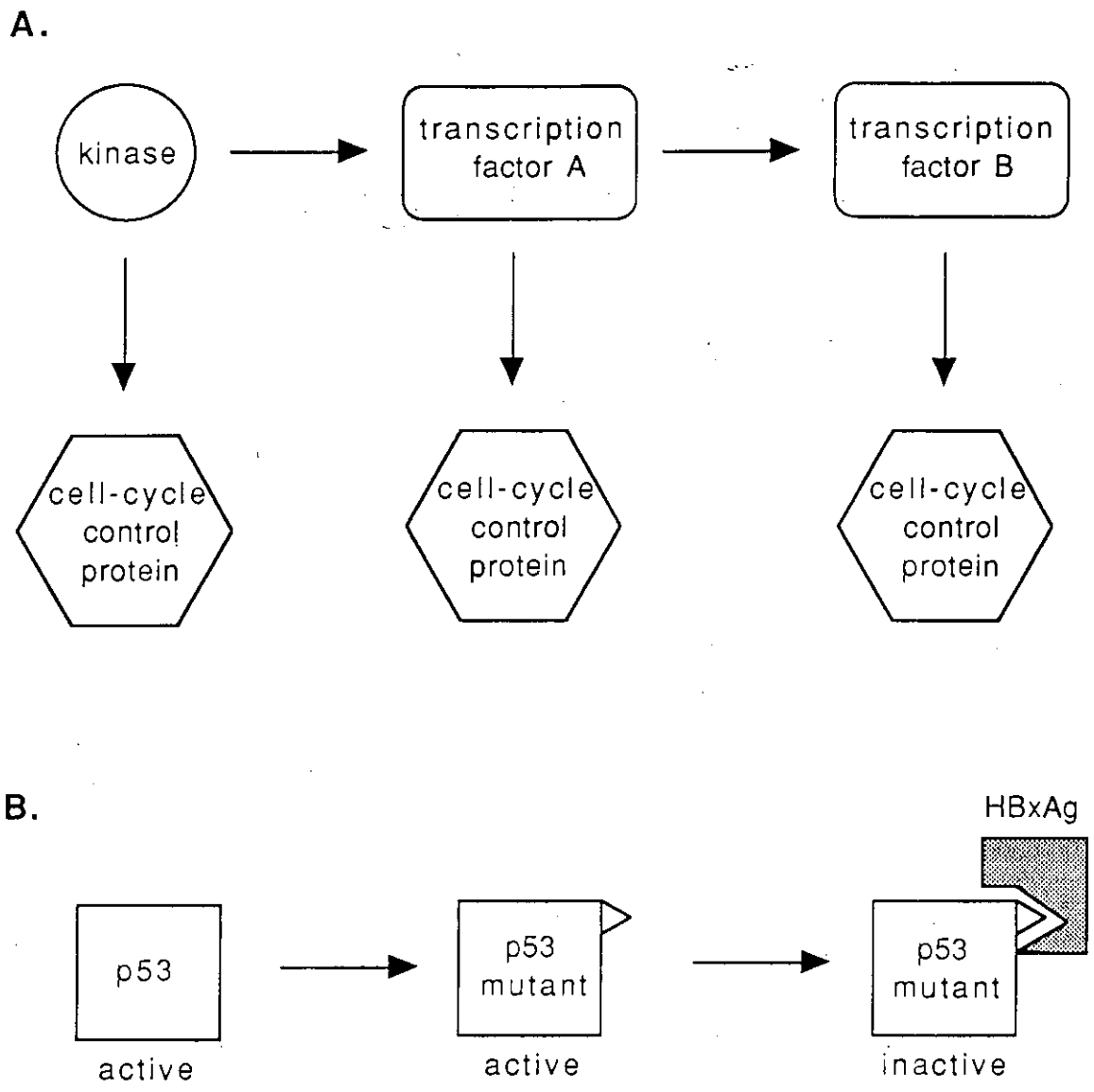


FIGURE 6.3 Potential mechanisms for the involvement of HBxAg in hepatocyte transformation

A) HBxAg may contribute to the development of HCC in its proposed capacity as a protein kinase or as a transcriptional activator. A kinase may modulate the activity of a cell-cycle control protein directly by phosphorylation or indirectly by phosphorylating a trancription factor and altering its activity. Trancription factor "A" may modulate the concentration a cell-cyle control protein directly by acting on its promoter or indirectly by acting on the promoter of transcription factor "B".

B) A specific mutation in p53 may confer the ability to interact with HBxAg thereby inactivating the tumour suppressor protein.

to increase its activity, or enhanced activity of a factor due to the presence of a viral transactivator could directly activate a cellular gene encoding a cell-cycle control protein or could activate transcription of another transcription factor which in turn activates a mitogenic gene (figure 6.3a). An example of the former type of target may be the IL-2 receptor gene whose transcription is activated by $p40^{tax}$ (Cross *et al.*, 1987). An example of the latter type of target may be the c-*myc* gene whose transcription is activated by HBxAg (Koike *et al.*, 1989) in the same HBxAg expression cell line which forms tumours when introduced into nude mice (Shirakata *et al.*, 1989). Unger and Shaul (1990) speculate that fusion of HBxAg coding sequence and host cellular sequence at the junction of integrated HBV DNA could affix a DNA binding domain to HBxAg creating a potent transcriptional activator.

While activation of cellular transcription factors could account for the potential role of HBxAg in development of HCC, the transformation of cells by adenovirus and SV40 provides an example of another possible mechanism. The adenovirus E1a protein is a viral transactivator capable of activating transcription of cellular genes, and it promotes oncogenic transformation. However, these two functions appear to be separable and distinct activities of the protein (reviewed by Flint and Shenk, 1989). A model for the transforming ability of E1a indicates that this function is mediated, at least in part, by it's ability to bind and functionally inactivate the retinoblastoma susceptibility gene product (Rb) (reviewed by Cooper and Whyte, 1989). The normal function of Rb is to negatively regulate retinoblast proliferation; both alleles of the *Rb*-gene are deleted or mutated in most retinoblastomas, and mutations in E1a that reduce its ability to bind Rb diminish its transforming activity. Recently, this sort of "anti-oncogene" function has been assigned to the p53 protein which was originally identified in mutant form at high levels in many tumours and tumour cell lines. Interaction of SV40 Tag with wild type p53 may be part of the mechanism by which cells are transformed upon infection with the virus (reviewed by Lane and Benchimol, 1990).

Very recently, two reports have emerged describing a mutational hotspot in p53 genes isolated from HCC tissues. In one study (Hsu *et al.*, 1991), 10/16 HCC tissue samples contained a mutant p53 gene; 50% of these mutations resulted in a specific amino acid change (residue 249; arginine to serine) present in either hemizygous or heterozygous configuration. In the other study (Bressac *et al.*, 1991) mutations in the p53 gene were detected in 5/10 HCC tissues examined. All of the p53 mutations were found in tumours from patients with current or earlier HBV infection. 60% of these mutations resulted in

the same amino acid change observed by Hsu *et al.* Bressac *et al.* note that 2/6 HCC-derived cell lines, including PLC/PRF/5, harboured the identical p53 mutation, while tumours of other cell types showed a range of p53-gene mutations none of which affected amino acid residue 249. Therefore, the mutation at 249 appears to be HCC specific and may, in association with HBV infection, be involved in progression of HCC.

The appearance of the specific mutation argues against this mutation being loss of function and implies a gain in function (Harris, 1991). One possible gain in function would be the ability to interact with an HBV encoded protein, perhaps HBxAg, which could functionally inactivate the mutant p53 molecule (figure 6.3b). Affinity purification of mutant p53 expressed in PLC/PRF/5 cells, known to express HBxAg (Moriarty *et al.*, 1985), would begin to provide insight into this possibility.

6C Concluding Remarks

The function of HBxAg as an activator of HBV surface antigen expression has been established in this study and is consistent with reports of concurrent investigations. This effect may be relevant to the very high levels of HBsAg secreted from naturally infected cells. Other roles for HBxAg transactivation in the progression of liver disease in individuals infected with HBV are under intensive investigation. The effect of HBxAg on viral and cellular transcription appears to be mediated through cellular factors, although the mechanism of this interaction remains to be elucidated. Investigation of this and other viral transcriptional regulatory mechanisms will provide insight into general features of eukaryotic gene expression.

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Appendix I: Nucleotide sequence of HBV subtype *adyw*

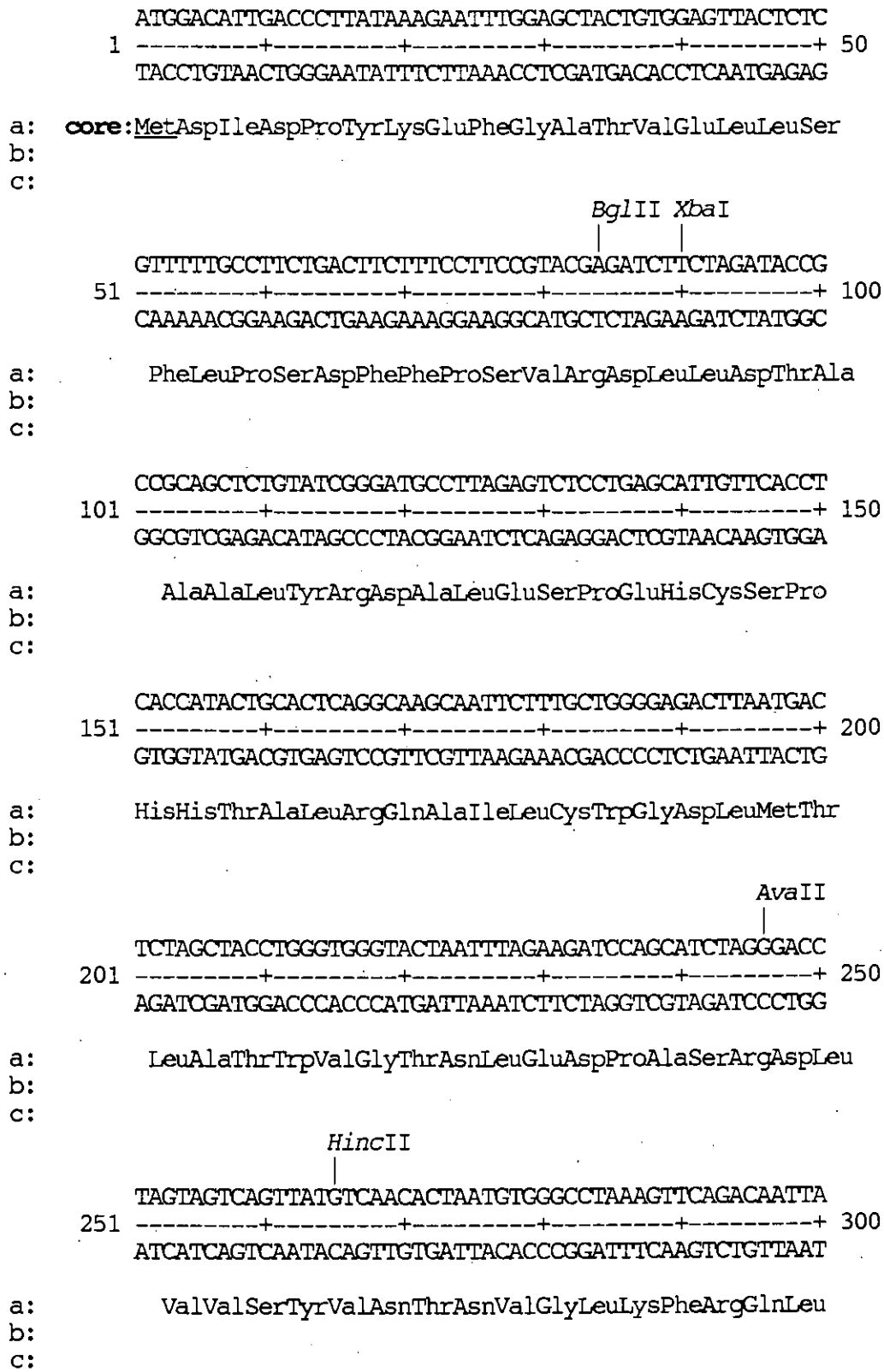
The nucleotide sequence of HBV subtype *adyw* contained in plasmid pHBV130 (Pasek *et al.*, 1979; Gough and Murray, 1982; Pugh *et al.*, 1986) is shown. Both strands of the complete genome (3182 nucleotides) are shown with the sense strand on top. Numbering is by the convention of Pasek *et al.* (1979), which sets number 1 at the start of the core ORF. Restriction endonuclease cleavage sites are indicated at the first nucleotide of the recognition sequence. The amino acid sequence of the viral ORFs is shown in their respective translational frames.

ORFs

preC	3096-3182	frame c
core	1-549	frame a
polymerase	407-2903	frame b
preS1	948-1271	frame c
preS2	1272-1436	frame c
surface	1437-2114	frame c
X	2656-3117	frame a

Recognition sites of restriction endonucleases used in these studies

*Ava*II	246, 461, 1421, 1464, 1791, 2854
*Bam*HI	1004, 2682
*Ban*II	1145, 2759
*Bcl*I	732
*Bgl*II	84, 499, 523
*Dra*I	2113, 3004
*Hinc*II	265, 1498, 2271, 2964
*Nco*I	2654
*Pst*I	1796
*Sph*I	2514
*Xba*I	90, 345, 1529
*Xho*I	1409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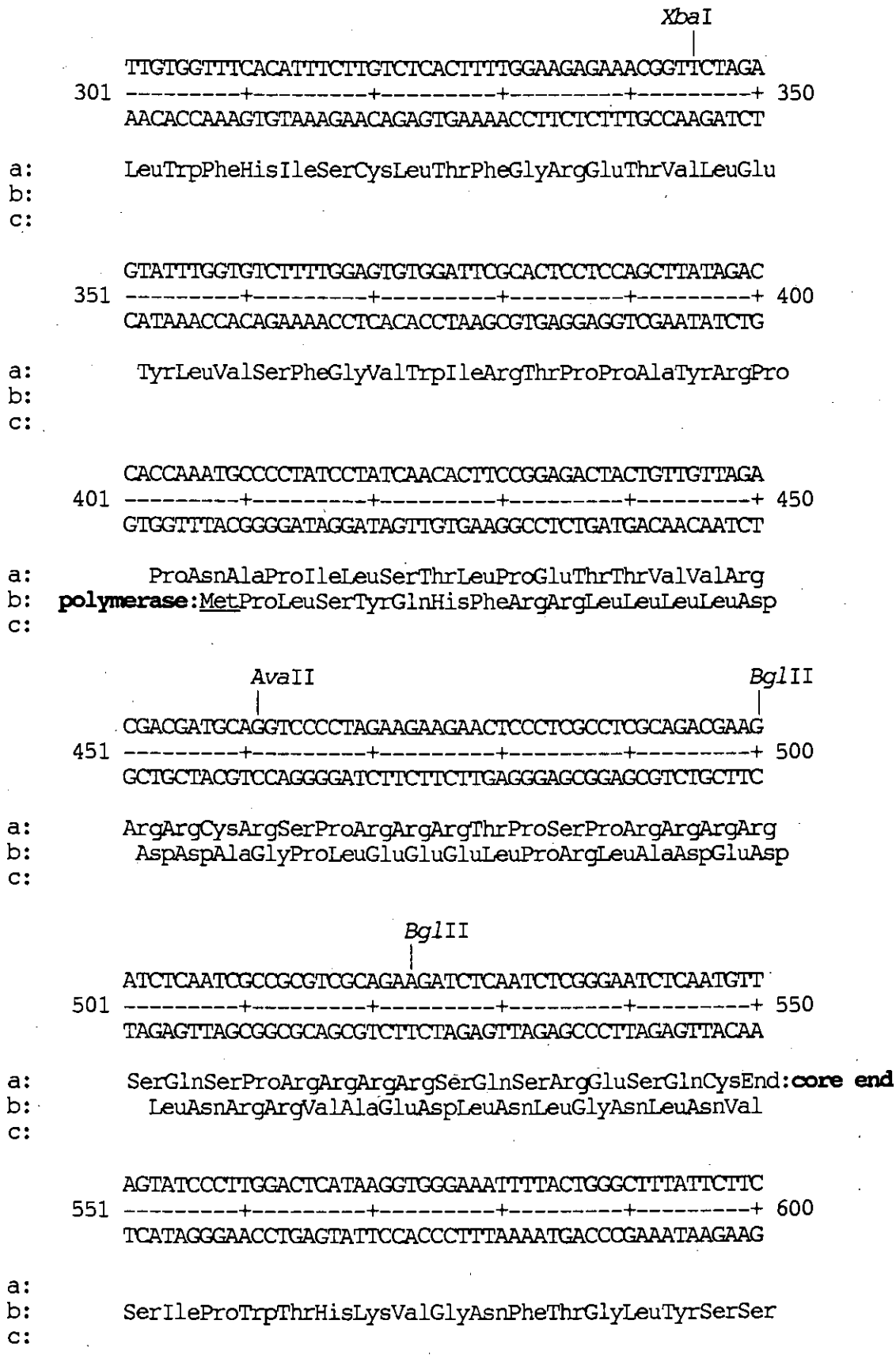
1
50
TACCTGTAACTGGGAATATTTCTTAAACCTCGATGACACCTCAATGAGAG
a: core:MetAspIleAspProTyrLysGluPheGlyAlaThrValGluLeuLeuSer
b:
c:
BglII XbaI
GTTTTTGCCTTCTGACTTCTTTCCTTCCGTACGAGATCTTCTAGATACCG
51
100
CAAAAACGGAAGACTGAAGAAAGGAAGGCATGCTCTAGAAGATCTATGGC
a: PheLeuProSerAspPhePheProSerValArgAspLeuLeuAspThrAla
b:
c:
CCGCAGCTCTGTATCGGGATGCCTTAGAGTCTCCTGAGCATTGTTCACCT
101
150
GGCGTCGAGACATAGCCCTACGGAATCTCAGAGGACTCGTAACAAGTGGA
a: AlaAlaLeuTyrArgAspAlaLeuGluSerProGluHisCysSerPro
b:
c:
CACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGAGACTTAATGAC
151
200
GTGGTATGACGTGAGTCCGTTCGTTAAGAAACGACCCCTCTGAATTACTG
a: HisHisThrAlaLeuArgGlnAlaIleLeuCysTrpGlyAspLeuMetThr
b:
c:
AvaII
TCTAGCTACCTGGGTGGGTACTAATTTAGAAGATCCAGCATCTAGGGACC
201
250
AGATCGATGGACCCACCCATGATTAAATCTTCTAGGTCGTAGATCCCTGG
a: LeuAlaThrTrpValGlyThrAsnLeuGluAspProAlaSerArgAspLeu
b:
c:
HincII
TAGTAGTCAGTTATGTCAACACTAATGTGGGCCTAAAGTTCAGACAATTA
251
300
ATCATCAGTCAATACAGTTGTGATTACACCCGGATTTCAAGTCTGTTAAT
a: ValValSerTyrValAsnThrAsnValGlyLeuLysPheArgGlnLeu
b:
c: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a: LeuTrpPheHisIleSerCysLeuThrPheGlyArgGluThrValLeuGlu
b:
c:
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351 ---------+---------+---------+---------+---------+ 400
CATAAACCACAGAAAACCTCACACCTAAGCGTGAGGAGGTCGAATATCTG
a: TyrLeuValSerPheGlyValTrpIleArgThrProProAlaTyrArgPro
b:
c:
CACCAAATGCCCCTATCCTATCAACACTTCCGGAGACTACTGTTGTTAGA
401 ---------+---------+---------+---------+---------+ 450
GTGGTTTACGGGGATAGGATAGTTGTGAAGGCCTCTGATGACAACAATCT
a: ProAsnAlaProIleLeuSerThrLeuProGluThrThrValValArg
b: polymerase:MetProLeuSerTyrGlnHisPheArgArgLeuLeuLeuLeuAsp
c:
AvaII
BglII
CGACGATGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG
451 ---------+---------+---------+---------+---------+ 500
GCTGCTACGTCCAGGGGATCTTCTTCTTGAGGGAGCGGAGCGTCTGCTTC
a: ArgArgCysArgSerProArgArgArgThrProSerProArgArgArgArg
b: AspAspAlaGlyProLeuGluGluGluLeuProArgLeuAlaAspGluAsp
c:
BglII
ATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTT
501 ---------+---------+---------+---------+---------+ 550
TAGAGTTAGCGGCGCAGCGTCTTCTAGAGTTAGAGCCCTTAGAGTTACAA
a: SerGlnSerProArgArgArgArgSerGlnSerArgGluSerGlnCysEnd:core end
b: LeuAsnArgArgValAlaGluAspLeuAsnLeuGlyAsnLeuAsnVal
c:
AGTATCCCTTGGACTCATAAGGTGGGAAATTTTACTGGGCTTTATTCTTC
551 ---------+---------+---------+---------+---------+ 600
TCATAGGGAACCTGAGTATTCCACCCTTTAAAATGACCCGAAATAAGAAG
a:
b: SerIleProTrpThrHisLysValGlyAsnPheThrGlyLeuTyrSerSer
c:

```
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      601 ---------+---------+---------+---------+---------+ 650
          ATGACATGGACAGAAATTGGGAGTAACCTTTTGCGGGAGAAAAGGATTAT

a:
b:          ThrValProValPheAsnProHisTrpLysThrProSerPheProAsnIle
c:

          TACATTTACACCAAGATATTATCAAAAAATGTGAACAGTTTGTAGGGCCG
      651 ---------+---------+---------+---------+---------+ 700
          ATGTAAATGTGGTTCTATAATAGTTTTTTACACTTGTCAAACATCCCGGC

a:
b:            HisLeuHisGlnAspIleIleLysLysCysGluGlnPheValGlyPro
c:

                                          BclI
                                           |
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      701 ---------+---------+---------+---------+---------+ 750
          GAGTGTCAGTTACTCTTTTCTTCCAATTTTAACTAGTACGGACGATCCAA

a:
b:          LeuThrValAsnGluLysArgArgLeuLysLeuIleMetProAlaArgPhe
c:

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      751 ---------+---------+---------+---------+---------+ 800
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a:
b:           TyrProAsnPheThrLysTyrLeuProLeuAspLysGlyIleLysProTyr
c:

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      801 ---------+---------+---------+---------+---------+ 850
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a:
b:            TyrProGluHisLeuValAsnHisTyrPheGlnThrArgHisTyrLeu
c:

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      851 ---------+---------+---------+---------+---------+ 900
          GTGTGAGATACCTTCCGCCCACAAAATATATTCTCTCATAGTTGTGTATC

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c:
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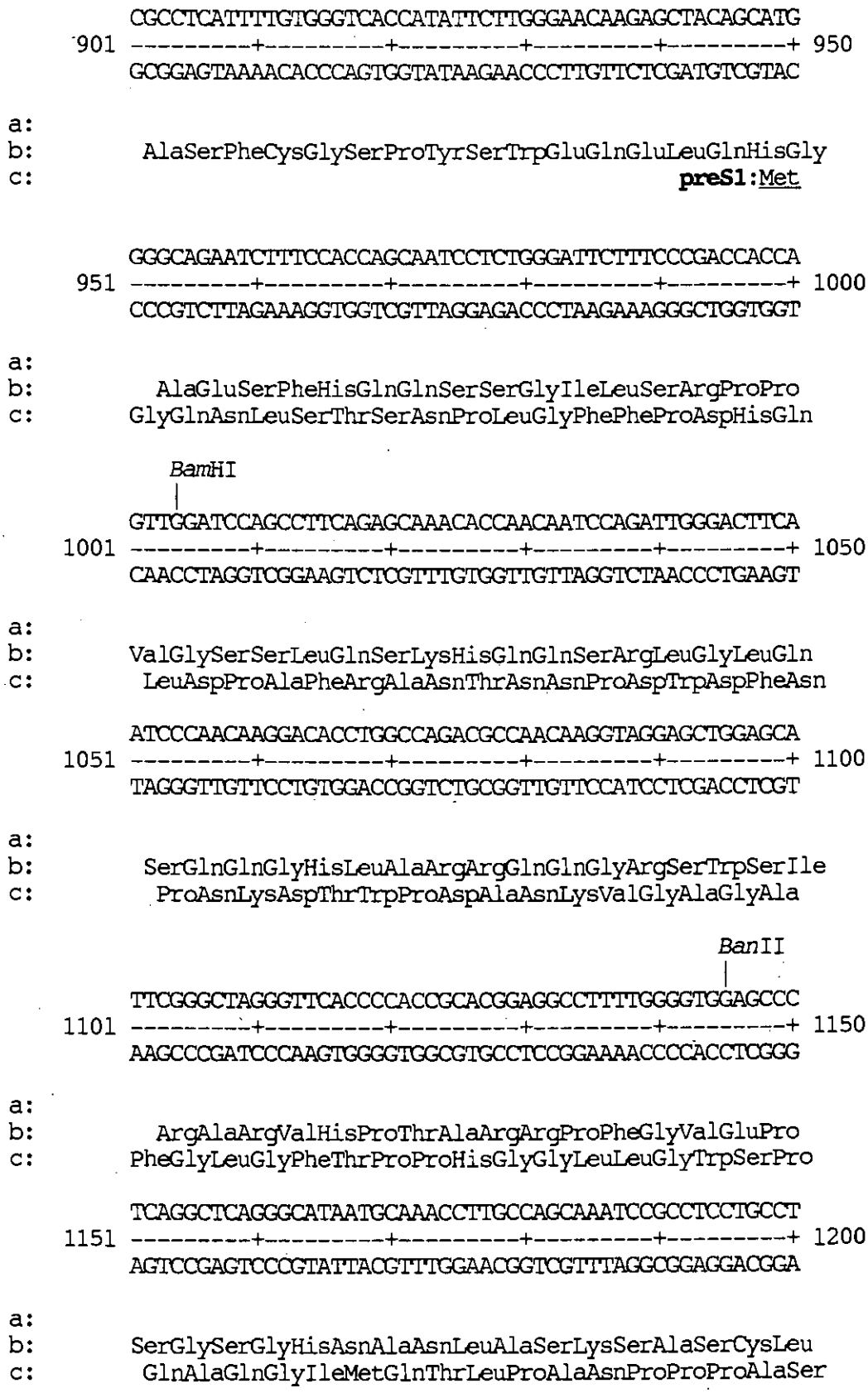
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a:
b: AlaSerPheCysGlySerProTyrSerTrpGluGlnGluLeuGlnHisGly
c: preS1:Met
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951 ---------+---------+---------+---------+---------+ 1000
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c: GlyGlnAsnLeuSerThrSerAsnProLeuGlyPhePheProAspHisGln
BamHI
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1001 ---------+---------+---------+---------+---------+ 1050
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a:
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c: LeuAspProAlaPheArgAlaAsnThrAsnAsnProAspTrpAspPheAsn
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1051 ---------+---------+---------+---------+---------+ 1100
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BanII
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1101 ---------+---------+---------+---------+---------+ 1150
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1151 ---------+---------+---------+---------+---------+ 1200
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a:
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c: GlnAlaGlnGlyIleMetGlnThrLeuProAlaAsnProProProAlaSer

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          1201  ---------+---------+---------+---------+---------+ 1250
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a:
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c:                 ThrAsnArgGlnSerGlyArgGlnProThrProLeuSerProProLeu

                AGAACCACTCATCCTCAGGCCATGCAGTGGAACTCCACAACCTTCCACCA
          1251  ---------+---------+---------+---------+---------+ 1300
                TCTTGGTGAGTAGGAGTCCGGTACGTCACCTTGAGGTGTTGGAAGGTGGT

a:
b:                  AsnHisSerSerSerGlyHisAlaValGluLeuHisAsnLeuProPro
c:                ArgThrThrHisProGlnAlaMetGlnTrpAsnSerThrThrPheHisGln
                             preS2:

                AACTCTGCAAGATCCCAGAGTGAGAGGCCTGTATTTCCCTGCTGGTGGCT
          1301  ---------+---------+---------+---------+---------+ 1350
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a:
b:                AsnSerAlaArgSerGlnSerGluArgProValPheProCysTrpTrpLeu
c:                 ThrLeuGlnAspProArgValArgGlyLeuTyrPheProAlaGlyGlySer

                CCAGTTCAGGGACAGTAAACCCTGTTCCGACTACTGCCTCTCCCATATCG
          1351  ---------+---------+---------+---------+---------+ 1400
                GGTCAAGTCCCTGTCATTTGGGACAAGGCTGATGACGGAGAGGGTATAGC

a:
b:                 GlnPheArgAspSerLysProCysSerAspTyrCysLeuSerHisIleVal
c:                  SerSerGlyThrValAsnProValProThrThrAlaSerProIleSer

                       XhoI         AvaII
                        |            |
                TCAATCTTCTCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCAC
          1401  ---------+---------+---------+---------+---------+ 1450
                AGTTAGAAGAGCTCCTAACCCCTGGGACGCGACTTGTACCTCTTGTAGTG

a:
b:                  AsnLeuLeuGluAspTrpGlyProCysAlaGluHisGlyGluHisHis
c:                SerIlePheSerArgIleGlyAspProAlaLeuAsnMetGluAsnIleThr
                                        surface:

                           AvaII                                  HincII
                             |                                      |
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          1451  ---------+---------+---------+---------+---------+ 1500
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a:
b:                IleArgIleProArgThrProAlaArgValThrGlyGlyValPheLeuVal
c:                 SerGlyPheLeuGlyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeu
```


XbaI
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a:
b: AspLysAsnProHisAsnThrAlaGluSerArgLeuValValAspPheSer
c: ThrArgIleLeuThrIleProGlnSerLeuAspSerTrpTrpThrSer
CTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTC
1551 ---------+---------+---------+---------+---------+ 1600
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a:
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c: LeuAsnPheLeuGlyGlyThrThrValCysLeuGlyGlnAsnSerGlnSer
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1601 ---------+---------+---------+---------+---------+ 1650
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1651 ---------+---------+---------+---------+---------+ 1700
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a:
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1701 ---------+---------+---------+---------+---------+ 1750
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a:
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AvaII PstI
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1751 ---------+---------+---------+---------+---------+ 1800
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a:
b: ArgLeuSerSerAsnSerArgIleIleAsnHisGlnHisGlyThrLeuGln
c: ValCysProLeuIleProGlySerSerThrThrSerThrGlyProCysArg

```
          GAACCTGCACGACTCCTGCTCAAGGAATCTCTATGTATCCCTCCTGTTGC
     1801 ---------+---------+---------+---------+---------+ 1850
          CTTGGACGTGCTGAGGACGAGTTCCTTAGAGATACATAGGGAGGACAACG

a:
b:          AsnLeuHisAspSerCysSerArgAsnLeuTyrValSerLeuLeuLeuLeu
c:           ThrCysThrThrProAlaGlnGlyIleSerMetTyrProSerCysCys

          TGTACAAAACCTTCGGATGGAAACTGCACCTGTATTCCCATCCCATCATC
     1851 ---------+---------+---------+---------+---------+ 1900
          ACATGTTTTGGAAGCCTACCTTTGACGTGGACATAAGGGTAGGGTAGTAG

a:
b:        LeuTyrLysThrPheGlyTrpLysLeuHisLeuTyrSerHisProIleIle
c:          CysThrLysProSerAspGlyAsnCysThrCysIleProIleProSerSer

          CTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCTT
     1901 ---------+---------+---------+---------+---------+ 1950
          GACCCGAAAGCCTTTTAAGGATACCCTCACCCGGAGTCGGGCAAAGAGAA

a:
b:          LeuGlyPheArgLysIleProMetGlyValGlyLeuSerProPheLeuLeu
c:           TrpAlaPheGlyLysPheLeuTrpGluTrpAlaSerAlaArgPheSerTrp

          GGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCC
     1951 ---------+---------+---------+---------+---------+ 2000
          CCGAGTCAAATGATCACGGTAAACAAGTCACCAAGCATCCCGAAAGGGGG

a:
b:          AlaGlnPheThrSerAlaIleCysSerValValArgArgAlaPheProHis
c:           LeuSerLeuLeuValProPheValGlnTrpPheValGlyLeuSerPro

          ATTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCT
     2001 ---------+---------+---------+---------+---------+ 2050
          TAACAAACCGAAAGTCAATATACCTACTACACCATAACCCCCGGTTCAGA

a:
b:           CysLeuAlaPheSerTyrMetAspAspValValLeuGlyAlaLysSer
c:          IleValTrpLeuSerValIleTrpMetMetTrpTyrTrpGlyProSerLeu

          GTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTC
     2051 ---------+---------+---------+---------+---------+ 2100
          CATGTCGTAGAACTCAGGGAAAAATGGCGACAATGGTTAAAAGAAAACAG

a:
b:          ValGlnHisLeuGluSerLeuPheThrAlaValThrAsnPheLeuLeuSer
c:           TyrSerIleLeuSerProPheLeuProLeuLeuProIlePhePheCysLeu
```

*Dra*I

TTTGGGCATACATTTAAACCCTAACAAAACAAAAAGATGGGGTTATTCTC
2101 ---------+---------+---------+---------+---------+ 2150
AAACCCGTATGTAAATTTGGGATTGTTTTGTTTTTCTACCCCAATAAGAG

a:
b: LeuGlyIleHisLeuAsnProAsnLysThrLysArgTrpGlyTyrSerLeu
c: TrpAlaTyrIleEnd:**surface end**

TAAATTTCATGGGCTATGTCATTGGATGTTGGGGATCATTGCCACAAGAT
2151 ---------+---------+---------+---------+---------+ 2200
ATTTAAAGTACCCGATACAGTAACCTACAACCCCTAGTAACGGTGTTCTA

a:
b: AsnPheMetGlyTyrValIleGlyCysTrpGlySerLeuProGlnAsp
c:

CACATCATACACAAAATCAAAGAATGTTTTAGAAAACTCCCTGTTCACAG
2201 ---------+---------+---------+---------+---------+ 2250
GTGTAGTATGTGTTTTAGTTTCTTACAAAATCTTTTGAGGGACAAGTGTC

a:
b: HisIleIleHisLysIleLysGluCysPheArgLysLeuProValHisArg
c:

*Hinc*II

GCCTATTGATTGGAAAGTGTGTCAACGAATTGTGGGTCTTCTGGGTTTTG
2251 ---------+---------+---------+---------+---------+ 2300
CGGATAACTAACCTTTCACACAGTTGCTTAACACCCAGAAGACCCAAAAC

a:
b: ProIleAspTrpLysValCysGlnArgIleValGlyLeuLeuGlyPheAla
c:

CTGCCCCTTTCACACAATGTGGTTATCCTGCTTTAATGCCCTTGTATGCA
2301 ---------+---------+---------+---------+---------+ 2350
GACGGGGAAAGTGTGTTACACCAATAGGACGAAATTACGGGAACATACGT

a:
b: AlaProPheThrGlnCysGlyTyrProAlaLeuMetProLeuTyrAla
c:

TGTATTCAATCTAAGCAGGCTTTCACTTTTTCGCCAACTTACAAGGCCTT
2351 ---------+---------+---------+---------+---------+ 2400
ACATAAGTTAGATTCGTCCGAAAGTGAAAAAGCGGTTGAATGTTCCGGAA

a:
b: CysIleGlnSerLysGlnAlaPheThrPheSerProThrTyrLysAlaPhe
c:


TCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCAGGCAACGGCCAG
2401 ---------+---------+---------+---------+---------+ 2450
AGACACATTTGTTATGGACTTGGAAATGGGGCAACGGTCCGTTGCCGGTC
a:
b: LeuCysLysGlnTyrLeuAsnLeuTyrProValAlaArgGlnArgProGly
c:
GTCTGTGCCAAGTGTTTGCTGATGCAACCCCCACTGGCTGGGGCTTGGTC
2451 ---------+---------+---------+---------+---------+ 2500
CAGACACGGTTCACAAACGACTACGTTGGGGGTGACCGACCCCGAACCAG
a:
b: LeuCysGlnValPheAlaAspAlaThrProThrGlyTrpGlyLeuVal
c:
SphI
ATGGGCCATCAGCGCATGCGTGGAACCTTTCTGGCTCCTCTGCCGATCCA
2501 ---------+---------+---------+---------+---------+ 2550
TACCCGGTAGTCGCGTACGCACCTTGGAAAGACCGAGGAGACGGCTAGGT
a:
b: MetGlyHisGlnArgMetArgGlyThrPheLeuAlaProLeuProIleHis
c:
TACTGCGGAACTCCTAGCCGCTTGTTTTGCTCGCAGCAGGTCTGGAGCAA
2551 ---------+---------+---------+---------+---------+ 2600
ATGACGCCTTGAGGATCGGCGAACAAAACGAGCGTCGTCCAGACCTCGTT
a:
b: ThrAlaGluLeuLeuAlaAlaCysPheAlaArgSerArgSerGlyAlaAsn
c:
ACATTCTCGGAACTGACAACTCTGTTGTCCTCTCCCGCAAATATACATCG
2601 ---------+---------+---------+---------+---------+ 2650
TGTAAGAGCCTTGACTGTTGAGACAACAGGAGAGGGCGTTTATATGTAGC
a:
b: IleLeuGlyThrAspAsnSerValValLeuSerArgLysTyrThrSer
c:
NcoI
BamHI
TTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTC
2651 ---------+---------+---------+---------+---------+ 2700
AAAGGTACCGACGATCCGACACGACGGTTGACCTAGGACGCGCCCTGCAG
a: X:MetAlaAlaArgLeuCysCysGlnLeuAspProAlaArgAspVal
b: PheProTrpLeuLeuGlyCysAlaAlaAsnTrpIleLeuArgGlyThrSer
c:


CTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCGGACGACCCTTCTCGGG
2701 ---------+---------+---------+---------+---------+ 2750
GAAACAAATGCAGGGCAGCCGCGACTTAGGACGCCTGCTGGGAAGAGCCC
a: LeuCysLeuArgProValGlyAlaGluSerCysGlyArgProPheSerGly
b: PheValTyrValProSerAlaLeuAsnProAlaAspAspProSerArgGly
c:
BanII
GCCGCTTGGGGCCCTGTCGTCCTCTTCTCTGCCTGCCGTTCCGGCCGCCC
2751 ---------+---------+---------+---------+---------+ 2800
CGGCGAACCCCGGGACAGCAGGAGAAGAGACGGACGGCAAGGCCGGCGGG
a: ProLeuGlyAlaLeuSerSerSerSerLeuProAlaValProAlaAlaHis
b: ArgLeuGlyProCysArgProLeuLeuCysLeuProPheArgProPro
c:
ACGGGGCGCACCTCTCTTTACGCGGCCTCCCCGTCTGTGCCTTCTCATCT
2801 ---------+---------+---------+---------+---------+ 2850
TGCCCCGCGTGGAGAGAAATGCGCCGGAGGGGCAGACACGGAAGAGTAGA
a: GlyAlaHisLeuSerLeuArgGlyLeuProValCysAlaPheSerSer
b: ThrGlyArgThrSerLeuTyrAlaAlaSerProSerValProSerHisLeu
c:
AvaII
GCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTCGCATGGAGACCAC
2851 ---------+---------+---------+---------+---------+ 2900
CGGCCTGGCACACGTGAAGCGAAGTGGAGACGTGCAGCGTACCTCTGGTG
a: AlaGlyProCysAlaLeuArgPheThrSerAlaArgArgMetGluThrThr
b: ProAspArgValHisPheAlaSerProLeuHisValAlaTrpArgProPro
c:
CGTGAACGCCCACCAAATCTTGCCCAAGGTCTTACATAAGAGGACTCTTG
2901 ---------+---------+---------+---------+---------+ 2950
GCACTTGCGGGTGGTTTAGAACGGGTTCCAGAATGTATTCTCCTGAGAAC
a: ValAsnAlaHisGlnIleLeuProLysValLeuHisLysArgThrLeuGly
b: End:polymerase end
c:
HincII
GACTCTCTGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGT
2951 ---------+---------+---------+---------+---------+ 3000
CTGAGAGACGTTACAGTTGCTGGCTGGAACTCCGTATGAAGTTTCTGACA
a: LeuSerAlaMetSerThrThrAspLeuGluAlaTyrPheLysAspCys
b:
c:

```
            DraI
             |
        TTGTTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAGATTAAAGGTCTT
3001    ---------+---------+---------+---------+---------+ 3050
        AACAAATTTCTGACCCTCCTCAACCCCCTCCTCTAATCTAATTTCCAGAA

a:      LeuPheLysAspTrpGluGluLeuGlyGluGluIleArgLeuLysValPhe
b:
c:

        TGTACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCA
3051    ---------+---------+---------+---------+---------+ 3100
        ACATGATCCTCCGACATCCGTATTTAACCAGACGCGTGGTCGTGGTACGT

a:       ValLeuGlyGlyCysArgHisLysLeuValCysAlaProAlaProCysAsn
b:
c:                                                pre-core:MetGln

        ACTTTTTCACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTGTTCAAG
3101    ---------+---------+---------+---------+---------+ 3150
        TGAAAAAGTGGAGACGGATTAGTAGAGAACAAGTACAGGATGACAAGTTC

a:        PhePheThrSerAlaEnd:X end
b:
c:       LeuPheHisLeuCysLeuIleIleSerCysSerCysProThrValGlnAla

        CCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
3151    ---------+---------+---------+-- 3182
        GGAGGTTCGACACGGAACCCACCGAAACCCCG

a:
b:
c:        SerLysLeuCysLeuGlyTrpLeuTrpGly
```

Appendix II: References for transactivation by HBxAg

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Appendix III

Rossner, M.T., Jackson, R.J. and Murray, K. (1990). Modulation of expression of the hepatitis B virus surface antigen gene by the viral *X*-gene product. *Proceedings of the Royal Society B (London)* **241**:51-58.

Modulation of expression of the hepatitis B virus surface antigen gene by the viral *X*-gene product

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SUMMARY

Human hepatoma cells (HepG2 and HUH7) transfected with a plasmid (*pHBV1004*) containing the transcription units for the major surface antigen (*S*) mRNA and the *X* mRNA of hepatitis B virus (HBV) secreted surface antigen (HBsAg) into the culture medium. A frameshift mutation in the *X* gene carried by *pHBV1004* greatly reduced HBsAg production by cells transfected with an equivalent amount of the mutant (*pHBV1004-B*). The mutation could be complemented by cotransfection with a plasmid (*pSV2HBX*) containing the *X* structural gene under control of the SV40 early promoter. HBsAg production by cells cotransfected with *pHBV1004-B* and *pSV2HBX* was equivalent to that in cells transfected with the parent plasmid (*pHBV1004*) alone. Levels of HBsAg production were directly related to the amount of S mRNA produced, showing that the *X*-gene product (HBxAg) can modulate expression of the *S* gene.

1. INTRODUCTION

Hepatitis B virus (HBV) has a partially double stranded DNA genome of 3.2 kilobases (kb) that carries four open reading frames (ORFs) including the *S* gene encoding the surface antigen and the *C* gene encoding the core antigen. The size of a third ORF and amino acid sequence similarities between its translation product and retroviral reverse transcriptases indicate that it encodes the viral polymerase. The fouth ORF encodes a protein of 154 amino acids and has been termed *X* because the function of its product in the viral life-cycle is not established.

There is strong evidence that the *X*-gene product (HBxAg) is expressed during viral infection and is immunogenic. Expression of an *X*-specific mRNA was demonstrated in mammalian cells transfected with HBV DNA (Gough 1983; Saito *et al.* 1986; Siddiqui *et al.* 1986; Simonsen & Levinson 1983). Direct evidence that the *X* gene encodes a protein was provided by its expression as a fusion protein in both prokaryotic and eukaryotic systems to yield an immunogenic product (Elfassi *et al.* 1986; Kay *et al.* 1985; Meyers *et al.* 1986; Moriarty *et al.* 1985; Pugh *et al.* 1986; Siddiqui *et al.* 1987). The *X* antigen, synthesized as a fusion protein in *Escherichia coli*, served as a reagent to detect antibodies to HBxAg in the sera of HBV infected individuals (Weber *et al.* 1988), and antisera raised against *X*-fusion proteins or against synthetic peptides representing segments of HBxAg were used to detect cross-reacting proteins in liver biopsies from HBV infected patients, in human hepatoma cells containing integrated HBV DNA (Moriarty *et al.* 1985), and in cells transfected with the complete HBV genome (Chang *et al.* 1987; Pugh *et al.* 1986).

The *X* ORF is conserved in all mammalian hepadnaviruses and amino acid sequence comparisons show two strong regions of similarity in all species (Colgrove *et al.* 1989) suggesting a conserved function for the *X*-gene products. A model for repliction of the HBV genome requires reverse transcription of an RNA intermediate in a manner analogous to retroviral genome replication (Summers & Mason 1982). All human retroviruses characterized to date encode polypeptides that activate transcription from the viral long terminal repeat (LTR) suggesting that one of the HBV ORFs may encode a transcriptional transactivator. Several studies have shown that HBxAg can indeed exert a transactivating function that appears to be quite promiscuous with many different targets for transactivation (Aufiero & Schneider 1990; Colgrove *et al.* 1989; Seto *et al.* 1988; Seto *et al.* 1990; Siddiqui *et al.* 1989; Spandau & Lee 1988: Twu & Robinson 1989; Twu & Schloemer 1987; Wollersheim & Hofschneider 1988; Zahm *et al.* 1988). Here we show that HBxAg can elevate expression of HBsAg in transfected hepatoma cells and suggest that corresponding activity *in vivo* may contribute to the high level of HBsAg expression observed in the course of natural infection (Cossart 1971).

2. MATERIALS AND METHODS

Cloning reagents and techniques

Manipulation of plasmid DNA was done essentially as described by Maniatis *et al.* (1982). Restriction enzymes, DNA polymerase preparations and DNA ligase were purchased from Boehringer Mannheim.

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Plasmid constructions

Plasmid *pHBV1004* containing the transcription units for the major surface mRNA and the *X* mRNA, was constructed as follows: HBV sequences (1004-3182/1-84, in the numbering system of Pasek *et al.* (1979)) were removed from the plasmid *pHBV130* (Gough & Murray 1982) by complete digestion with *Bgl*II followed by partial digestion with *BamH*I. The resulting 2.3 kb fragment was ligated into the *BamH*I site of *pUC8*. Plasmid *pHBV1004-B* contains a frameshift mutation that was introduced within the *X* coding sequence in *pHBV1004* by partial digestion with *BamH*I followed by filling in the cohesive ends of the site at position 2682 with DNA polymerase I (Klenow fragment). To confirm that the major surface promoter sequences remained identical in the parent and mutant plasmids, DNA sequencing was done on the HBV sequences from positions 1004–1409 contained in the two plasmids with the dideoxy-nucleotide chain termination method (Sanger *et al.* 1977).

Plasmid *pSV2HBX* was constructed to express HBxAg. Plasmid *pSV2βG* (a gift from P. Southern) contains the rabbit β-globin gene under control of the SV40 early promoter/enhancer complex. The rabbit β-globin structural sequences were removed by digesting with *Hind*III, filling in the cohesive ends with DNA polymerase I (Klenow fragment) and digesting with *Bgl*II. The *X* gene (2654-3182/1-84) was cleaved from *pHBV130* by digesting with *Nco*I, filling in the cohesive ends with T4 DNA polymerase and digesting with *Bgl*II. The resulting 12 base pair (b.p.) fragment was ligated to the 4.2 kb vector fragment of *pSV2βG*. Plasmid *pSV2HBX-B* contains the same frameshift mutation in the *X* gene as *pHBV1004-B*, and was constructed by an equivalent series of reactions.

Plasmid DNA was prepared from cells (*E. coli* strain ED8654) harbouring the plasmid by lysis in alkaline-SDS (Maniatis *et al.* 1982), followed by several rounds of phenol extraction.

Cell culture

Human hepatoma cells devoid of integrated HBV sequences, HepG2 (Knowles *et al.* 1980) and HUH7 (Nakabayashi *et al.* 1982), were grown in Glasgow's modification of Eagles Medium (GMEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids and 10% (by volume) foetal calf serum (FCS) at 37 °C in 6% CO_2.

Transfection of hepatoma cells

Hepatoma cells were transfected with expression vectors by using the DEAE-Dextran/chloroquine method (Luthman & Magnusson 1983). Briefly, 4×10^6 cells were seeded onto 100 mm diameter plates 1 day before transfection. Plasmid DNA was added in 5 ml of a solution of serum-free GMEM containing 200 μg ml^{-1} DEAE-Dextran (molecular mass 5×10^5; Sigma), 50 mM Tris-HCl pH 7.3. DNA was adsorbed for 5 h (HUH7 cells) or 6 h (HepG2 cells) at 37 °C at which time chloroquine diphosphate (Sigma) was added to 100 μM. Cells were incubated for a further 2 h (HepG2) or 3 h (HUH7) after which the transfection solution was removed, cells were washed in PBS and incubated in GMEM supplemented with 10% (by volume) FCS for $3\frac{1}{2}$–$4\frac{1}{2}$ days.

Detection of HBsAg

HBsAg was detected by radioimmunoassay of the cell culture medium with the AUSRIAII-125 kit (Abbot Laboratories) according to the manufacturer's instructions. In some cases HBsAg was concentrated from the culture medium by ultracentrifugation (150000 *g* for 16 h) and resuspension in 1/30th volume of PBS for assay. For detection of HBsAg particles containing middle S (preS2/S) polypeptides, polystyrene beads were incubated in a solution of 0.2 M $NaHCO_3$, pH 9.2, containing 60 μg ml^{-1} anti-preS2 monoclonal antibody Q 19/10 (Heerman *et al.* 1988) at room temperature for at least 4 h. Beads were dried on absorbent paper and stored at 4 °C.

RNA extraction from transfected cells

RNA was extracted by a modification of the hot phenol method. Harvested cells were resuspended in 10 cell-volumes of 10 mM Tris-HCl, pH 7.4, 150 mM, NaCl, 3 mM $MgCl_2$, 1 mM EDTA, (pH 8.0). NP40 was added to 0.5% (by volume), cells were vortexed for 30 s and the lysate cleared by centrifugation (1600 *g*, 15 min) SDS was added to 0.5% (w/v) and the lysate was extracted with an equal volume of phenol at 60 °C. RNA was precipitated with ethanol.

Analysis of mRNA by hybridization

RNA preparations were treated with DNase and fractionated according to size by electrophoresis in 1% agarose gels containing 0.27 M formaldehyde. RNA was transferred to nylon filters (Hybond-N, Amersham) by strandard blotting methods (Maniatis *et al.* 1982) and was hybridized to [^{32}P]-labelled probes prepared by the random priming method (Feinberg & Vogelstein 1983). Hybridized filters were washed 2×15 min in $2 \times$ SSC, 0.1% SDS at room temperature and 1 h in $0.1 \times$ SSC, 0.1% SDS at 65 °C. Hybridizing RNAs were detected by autoradiography at −70 °C with an intensifying screen.

3. RESULTS

Plasmids carrying the coding sequences for HBxAg and HBsAg

Hepatitis-B-virus sequences used in this study were derived from plasmid *pHBV130* (Gough & Murray 1982), which contains a complete copy of the HBV genome subtype *adyw* (figure 1*a*). Plasmid *pHBV1004* was constructed to contain the transcription units for the major surface mRNA and *X* mRNA under control of their own expression elements (figure 1*b*). Plasmid *pHBV1004-B* contains a frameshift mutation that was introduced following the triplet coding for amino acid residue 10 (asp) within the *X* coding sequence in *pHBV1004*. Plasmid *pSV2HBX* was constructed to

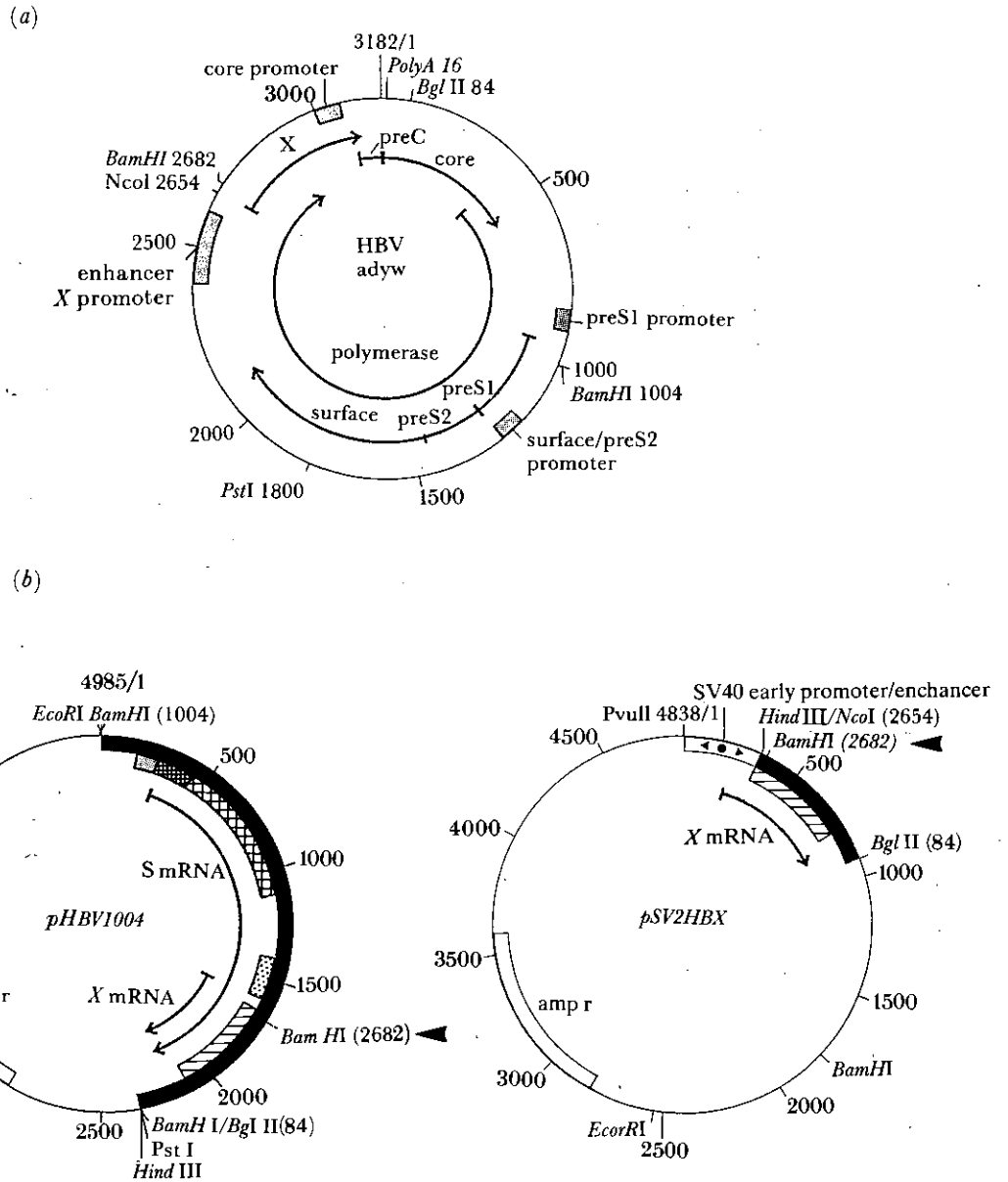


Figure 1. (*a*) Genomic organization of HBV subtype *adyw* used in this study. Open reading frames are represented by arrows. Boxes represent transcriptional control regions. Restriction sites used in this study are indicated. (*b*) The plasmids used in transfection experiments. Numbers in parentheses indicate the positions of the restriction sites in the HBV genome. Transcripts are represented by arrows inside the circles. The heavy arrows outside the circles indicate the site of the frameshift mutation in the *X* gene in *pHBV1004-B* and *pSV2HBX-B*. Key to boxes: (■), HBV DNA insert; (□), preS2/S promoter; (▦), pre-S2 ORF; (⊞), S ORF; (□), enhancer/*X* promoter; (▨). *X* ORF. (Construction of the plasmids is described in Materials and methods.)

express HBxAg under control of the SV40 early promoter and enhancer, and plasmid *pSV2HBX-B* contains the same frameshift mutation in the *X* gene as *pHBV1004-B* (figure 1*b*).

Frameshift mutation in the X gene reduces transient expression of HBsAg

Hepatoma cells (HepG2 or HUH7) transfected with *pHBV1004* secreted HBsAg into the culture medium where it was detected 4–5 days post-transfection by radio-immunoassay. Transfection with an equivalent amount of *pHBV1004-B* resulted in significant reduction in HBsAg synthesis (figure 2). The difference in HBsAg expression between the two plasmids was greatest when small amounts of DNA were used for transfection; HBsAg secretion by HepG2 cells transfected with 3 μg of *pHBV1004* was an average of fivefold (and as much as sevenfold) higher than by cells transfected with 3 μg of *pHBV1004-B*, but with 10 μg or 30 μg of the plasmids the difference was two-threefold. With the larger amounts of DNA the availability of transcription factors other than HBxAg may become limiting. Although it is possible, it is unlikely that the stability of the S mRNA was affected by the 4 b.p. insertion in the *X* coding sequence, for it has been shown that even a 270 b.p. deletion of the HBV enhancer contained within the S transcript did not affect mRNA stability in COS cells (Bulla & Siddiqui 1988).

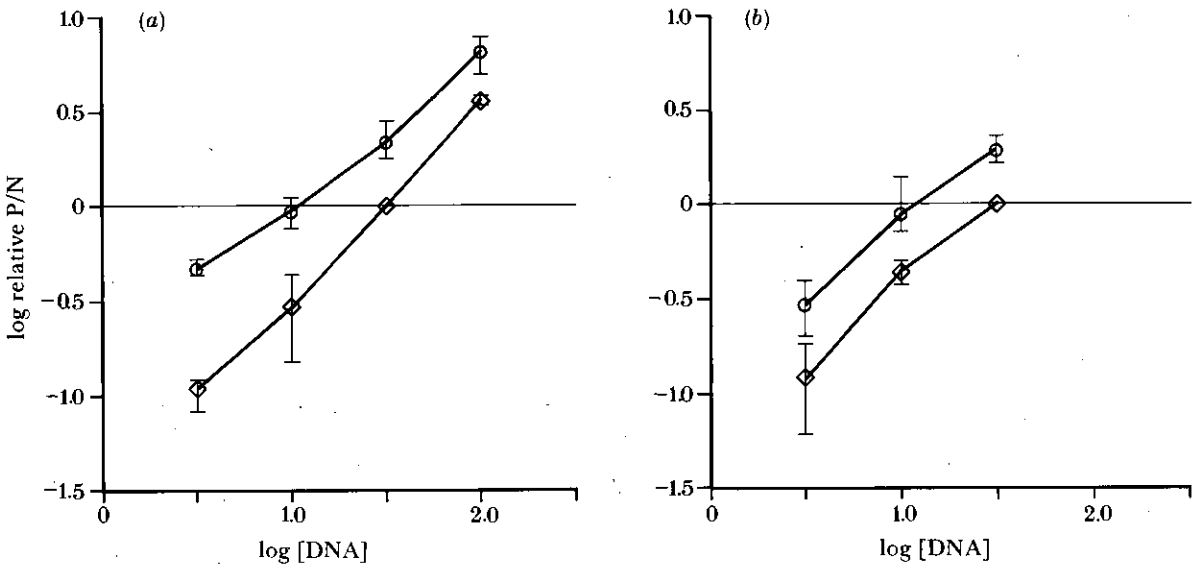


Figure 2. HBsAg production. Levels of HBsAg production from cells transfected with HBsAg expression plasmids. HBsAg was detected by radio-immunoassay of either the cell culture medium or medium that had been concentrated 30-fold to bring the values within the linear range of the assay. The level of HBsAg expression was initially calculated as a ratio of counts per minute obtained for the sample to counts per minute obtained from the medium of mock transfected cells (P/N). The P/N values within each experiment were then normalized to the P/N value obtained for cells transfected with 30 μg of *pHBV1004-B* (relative P/N value). This allows comparison of the values obtained in different experiments in which the absolute level of HBsAg expression may differ. The average relative P/N values were calculated by weighting the relative P/N values in each experiment according to the number of plates used for transfection with each amount of DNA. Error bars indicate the range of average values obtained in each experiment; (*a*) HepG2 cells; (*b*) HUH7 cells; (○), cells transfected with *pHBV1004*; (◇), cells transfected with *pHBV1004-B*.

Table 1. *PreS2 epitopes on HBsAg preparations*

source of HBgAg	P/N values[a] *AUSRIA-II125*	*preS2*
serum[b]	24.2	11.1
	24.8	7.5
yeast[c]	25.9	1.2
	24.9	1.1
HepG2 cells transfected with *pHBV1004*[d]	16.8	5.7
	20.5	5.3
HepG2 cells transfected with *pHBV1004-B*	5.8	1.8
	6.4	1.3

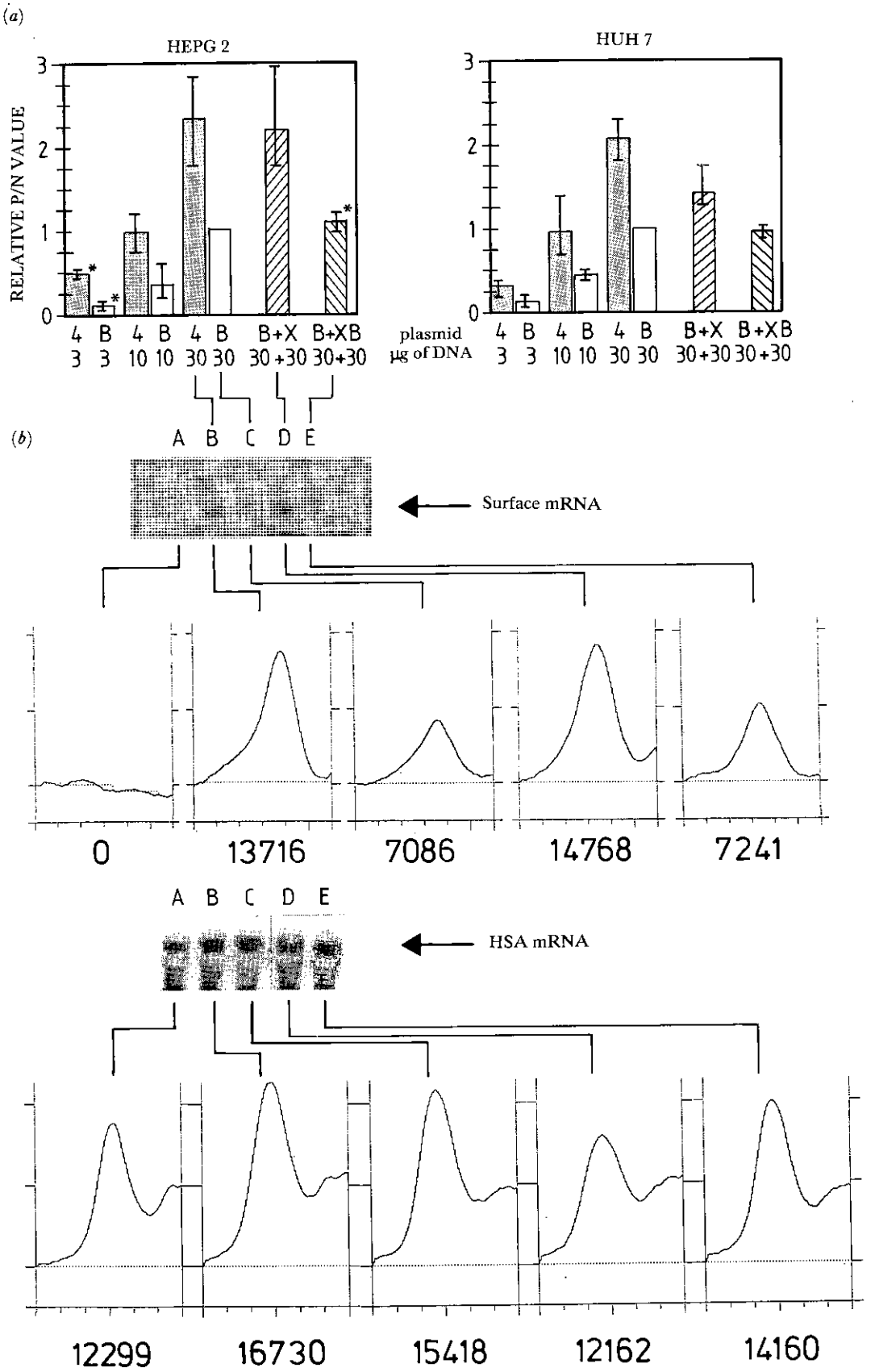
[a] Values are shown from two independent experiments.
[b] HBsAg derived from serum (20 ng ml^{-1}) was provided with the AUSRIA-II125 kit as a positive control.
[c] HBsAg derived from yeast was purified from *Sacchatomyces cerevisiae* harbouring plasmid *pHING2* (Murray *et al.* 1984), which contains the S coding region under control of the yeast *PHO5* promoter.
[d] *pHBV1004* and *pHBV1004-B* preparations are 1:30-fold concentrated medium from HepG2 cells transfected with 30 μg of the indicated plasmid.

The number of surface antigen particles containing middle S (preS2/S) polypeptides was detected by radio-immunoassay in which the solid phase was a polystyrene bead coated with anti-preS2 monoclonal antibody Q 19/10 (Heerman *et al.* 1987) and the bounded antigen was detected with [^{125}I]-labelled human anti-S serum provided with the AUSRIA II-125 HBsAg detection kit. Table 1 shows that transfection of hepatoma cells with *pHBV1004* resulted in the secretion of a greater number of HBsAg particles containing detectable preS2 epitopes than transfection with an equivalent amount of *pHBV1004-B*. The proportion of secreted HBsAg particles containing detectable preS2 epitopes was similar with both the parent and mutant plasmids. Assuming that the proportion of middle S polypeptides on each HBsAg particle was the same for those expressed from either the parent or mutant plasmid, transfection of cells with the mutant plasmid gave reduced expression of middle S polypeptides.

Complementation of the mutation in the X gene

The frameshift mutation in the *X* coding sequence contained in *pHBV1004-B* could be complemented by supplying HBxAg in *trans*. In these experiments, hepatoma cells were transfected with 30 μg of *pHBV1004-B*. This gave a level of HBsAg expression

Figure 3. (*a*) HBsAg production. Levels of HBsAg production from cells transfected with HBsAg expression plasmids or cotransfected with HBsAg plasmid and HBxAg expression plasmid. Calculation of the Relative P/N Value is described in figure 2 legend. Except where indicated, the values presented here are an average from at least three experiments (* indicates values that are an average from two experiments). Error bars indicate the range of average values obtained in each experiment. Key to plasmids: 4 = *pHBV1004*; B = *pHBV1004-B*; X = *pSV2HBX*; XB = *pSV2HBX-B*; 'μg of DNA' is the amount of DNA used to transfect each plate of cells. (*b*) Northern blot of RNA



from cells expressing HBsAg. RNA was prepared from three plates of cells, four days post-transfection with (*a*) 30 μg of *pUC8* per plate; (*b*) 30 μg of *pHBV1004*; (*c*) 30 μg of *pHBV1004-B*; (*d*) 30 μg of *pHBV1004-B* plus 30 μg of *pSV2HBX*; (*e*) 30 μg of *pHBV1004-B* plus 30 μg of *pSV2HBX-B*. the surface transcript probe was a 796 b.p. *BamHI*/*Ps*I fragment from *pHBV1004* representing HBV DNA positions 1004–1800. The probe for the *HSA* gene was a 1.8 kb *BamHI* fragment from the plasmid *p1243*, which contains the HSA gene under control of the caseine promoter. Densitometric analysis of the exposed X-ray film was done on the Shimadzu Dual Wavelength Cromato Scanner, Model CS-930. The numbers under the curves represent the area under each peak.

that could readily be assayed and that was about half that from the parent plasmid, *pHBV1004*. Cotransfection of HepG2 cells with *pHBV1004-B* and a plasmid carrying the *X* coding sequence under control of the SV40 early promoter and enhancer (*pSV2HBX*) restored HBsAg production to a level similar to that produced by cells transfected with an equivalent amount of *pHBV1004* (figure 3*a*). In HUH7 cells, HBsAg production from plasmid *pHBV1004-B* was increased 1.5-fold upon cotransfection with *pSV2HBX*. This may reflect a greater susceptibility to toxic effects of expression of HBxAg from a heterologous promoter that have been observed in CHO cells (J. Barsoum, personal communication).

Co-transfection can give rise to a concatanated network of input DNA molecules, and thus it was possible that the observed effect was due to a *cis* effect of the SV40 transcriptional control signals contained in *pSV2HBX*. As a control, hepatoma cells were co-transfected with *pHBV1004-B* and *pSV2HBX-B*, which contained the same frameshift mutation in the *X* coding sequence. HBsAg expression was then equivalent to that of cells transfected with *pHBV1004-B* alone (figure 3*a*). This established that the restoration of HBsAg expression to wild-type levels by co-transfection with the plasmid *pSV2HBX* was due to a *trans* effect of HBxAg.

HBxAg modulates the level of S mRNA

Total cellular RNA from cells transfected with HBsAg expression plasmids and from cells co-transfected with *pHBV1004-B* and the *X* expression plasmid or its corresponding frameshift mutant was fractionated by agarose gel electrophoresis and blotted to a nylon filter. Hybridization to a DNA probe containing sequences complementary to the S transcript revealed that the level of 2 kb S mRNA corresponded to the amount of HBsAg produced (figure 3*b*). The S transcript probe was stripped from the filter, which was then rehybridized with a [^{32}P]-labelled fragment from the plasmid *p1243*, a probe for human serum albumin (HSA) transcripts. The densitometer traces in the lower part of figure 3*b* showed that the samples analysed contained equivalent amounts of mRNA; all the values lie within -13% to $+18\%$ of the mean, the reproducibility of individual scans of the various bands being $\pm 7\%$.

4. DISCUSSION

We have shown that HBxAg can function to increase transient expression of HBsAg in two different human hepatoma cell lines. A frameshift mutation close to the 5′ end of the *X* coding sequence in the HBsAg expression vector reduced the amount of HBsAg secreted into the culture medium to as little as 14% of that with the parent plasmid. This mutation could be complemented by supplying HBxAg in *trans*, which is definitive evidence that HBxAg can modulate expression of another viral gene product.

The level of HBsAg expression corresponded to the amount of S mRNA (figure 3) indicating that HBxAg does not exert its effect at the level of translation or secretion. This effect is unlikely to be post-transcriptional as the S mRNA is not spliced and thus HBxAg cannot function like the *rev* protein of HIV in diverting transcripts from splicing (Felber *et al.* 1989; Malim *et al.* 1989). Furthermore, with the bacterial chloramphenicol acetyl transferase (CAT) gene as a reporter for promoter activity, HBxAg has been shown to affect transcription, but not mRNA stability (Siddiqui *et al.* 1989). Our results are also consistent with the recent demonstration that a mutation in the *X* gene contained within the complete HBV genome led to a reduction in the levels of both the pregenomic RNA and the S mRNA upon transient expression in HUH7 cells (Colgrove *et al.* 1989).

Several other transcriptional regulatory sequences, including retroviral LTRs, the SV40 early promoter/enhancer complex, the β-interferon promoter and the RNA polymerase III promoter of the Adenovirus2 VA1 RNA are targets for transactivation by HBxAg (Aufiero & Schneider 1990; Colgrove *et al.* 1989; Seto *et al.* 1988; Siddiqui *et al.* 1989; Spandau & Lee 1988; Twu & Schloemer 1987; Twu & Robinson 1989; Wollersheim & Hofschneider 1988; Zahm *et al.* 1988). The function of HBxAg therefore may be mediated through a common cellular transcription factor either by increasing its rate of expression or by interacting with the factor itself; in *Drosophila* cells, HBxAg can interact with human AP2 transcription factor to increase transcription from a reporter plasmid carrying multiple AP2 sites in the target promoter (Seto *et al.* 1990).

Four distinct cellular factors interacting with the HBV enhancer region are present in several cell types (Ben-Levy *et al.* 1989). This enhancer is a target for transactivation by HBxAg in association with the HBV core promoter or *X* promoter linked to CAT (Colgrove *et al.* 1989; Siddiqui *et al.* 1989; Spandau & Lee 1988). The HBV enhancer also activates transcription from the major, preS2/S promoter linked to CAT (Antonucci & Rutter 1989; Chang & Ting 1989; De-medina *et al.* 1988; Faktor *et al.* 1988; Siddiqui *et al.* 1986), and increases the transient expression of HBsAg from this promoter in HepG2 cells (Bulls & Siqqiqui 1988). This is similar to the requirement of the SV40 enhancer for efficient transcription from the SV40 major late promoter (Ernoult-Lange *et al.* 1984). The effect of HBxAg on the transient expression of HBsAg may thus be mediated through the HBV enhancer.

The HBsAg expression vector used in this study contained only the preS2/S promoter and not the TATA-like, preS1 promoter, which is located 300 b.p. upstream and directs synthesis of the 2.4 kb mRNA encoding the large preS1/preS2/S polypeptide. Although the two promoters in isolation have similar transcriptional activities (Antonucci & Rutter 1989; Siddiqui *et al.* 1986), the preS2/S promoter accounts for 98% of surface gene transcription in infected liver (Ou & Rutter 1985). Bulla & Siddiqui (1989) have described a negative regulatory region within the preS1 ORF that, when removed, greatly increases transcription from the preS1 promoter; all of the constructs used in their study expressed *X* mRNA,

however, and the role of HBxAg in this regulation is therefore unclear. Preliminary studies in this laboratory (M. T. Rossner & K. Murray, unpublished observations) indicate that HBxAg effectively transactivates the preS1 promoter and does not appear to be involved in the negative regulation described by Bulla & Siddiqui (1989).

The role of HBxAg in the viral life-cycle is still under intensive investigation. The effect reported here on HBsAg production may be relevant to the very high levels of HBsAg secretion from naturally infected hepatocytes. The secreted forms are predominantly 22 nm empty viral envelopes that may serve as 'decoy virions' to bind neutralizing antibodies produced in response to infection.

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